QUANTITATIVE ANALYSIS OF ANTIGEN-MEDIATED CD4 T CELL – CD4 T CELL COOPERATION DETERMINING THE TH1/TH2 PHENOTYPE OF A PRIMARY IMMUNE RESPONSE

A Thesis Submitted to the College of Graduate Studies and Research in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy in the Department of Microbiology and Immunology
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Abstract

Several variables have been found to affect the Th1/Th2 differentiation of newly activated CD4 T cells. This phenotype can be critical in determining effectiveness of immune responses. Experiments in this thesis were undertaken to better define the *in-vivo* cellular interactions involved in determining the Th1/Th2 phenotype of newly activated CD4 T cells.

Lethally irradiated BALB/c mice reconstituted with a constant number of syngeneic, naive spleen cells were challenged with xenogeneic red blood cells (XRBC) conjugated to ovalbumin (OVA) and the Th1/Th2 phenotype of the anti-XRBC response assessed. Antigen-specific interferon-gamma (IFN-γ) and interleukin-4 (IL-4) secreting cells obtained from spleens of immunized mice were enumerated by an ELISPOT assay; the relative number of IFN-γ- and IL-4-producing cells is taken as a relative measure of Th1 and Th2 components of the response. When challenged with a 'standard' dose of XRBC-OVA, predominant Th1 responses are generated; when challenged with a ten-fold lower dose, such reconstituted mice do not generate significant responses. This adoptive transfer system was employed to explore further the relationships between quantitative changes in the dose of immunizing antigen and the number of responding antigen-specific CD4 T cells, and the Th1/Th2 phenotype of immune responses generated. Unprimed transgenic CD4 T cells specific for OVA can modulate the Th1/Th2 phenotype of the anti-XRBC response upon immunization with XRBC-OVA. Addition of a small number of naive transgenic spleen cells to the standard

reconstituting population of normal spleen cells results in the generation of significant numbers of SRBC-specific Th2 cells when mice are challenged with a 'standard' dose, or can generate predominant Th1 responses when mice are challenged with a ten-fold lower dose. Transgenic cells only impact the Th1/Th2 phenotype of CD4 T cells specific for XRBC when OVA is linked to the XRBC. That CD4 T cells specific for different antigens cooperate only through the recognition of linked antigenic determinants has important implications for many aspects of immune regulation. Observations further show that thymocytes from transgenic mice can influence the XRBC-specific response phenotype in an identical manner as transgenic spleen cells, suggesting that previously polarized pro-Th1/Th2 cells are not required in the cooperative events influencing Th1/Th2 phenotype of newly activated CD4 T cells.

These observations lead to a quantitative description, whereby antigen-mediated CD4 T cell cooperation can affect the Th1/Th2 phenotype of a primary antigen-specific immune response, and provide a context for further analysis at the molecular level.

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Abbreviations

Ab	antibody
Ag	antigen
APC	antigen presenting cell
ATCC	American type culture collection
BCR	B cell receptor
BGG	bovine gamma globulin
BSA	bovine serum albumin
C’	complement
CD 80	B-7.1
CD 86	B-7.2
CD154	CD40L
CFA	complete Freund’s adjuvant
CFSE	carboxyfluorescein diacetate succinimidyl ester
CTL	cytotoxic T lymphocytes
CXCR	C-X-C chemokine receptor
DTH	delayed type hypersensitivity
DCs	dendritic cells
DNP	2,4-dinitro-phenol
ELISPOT	enzyme-linked immunospot assay
FACS	fluorescence-activated cell sorting
FASL	FAS ligand
FCS	fetal calf serum
FITC	fluorescein isothiocyanate
GvH	graft versus host
HEL	hen egg lysozyme
HGG	human gamma-globulin
HLA	(human) histocompatibility leukocyte antigen
IFN-□	interferon-gamma

Ig	immunoglobulin
IL	interleukin (for example, IL-2)
i.d.	intradermal
i.p.	intraperitoneal
i.v.	intravenous
KLH	keyhole limpet hemocyanin
LPS	lipopolysaccharide
TLR	TOLL-like receptor
MACS	magnetic-activated cell sorting
MHC	major histocompatibility complex
MLR	mixed lymphocyte reaction (one way)
□L	microlitre
OVA	ovalbumin
PCC	pigeon cytochrome c
PBS	phosphate-buffered saline
PBST	phosphate-buffered saline containing tween 20
PE	phycoerythrin
PLL	poly-l-lysine
PPD	purified protein derivative (of *M. tuberculosis*)
RAG	recombination activation gene(s)
RBC	red blood cell(s) (i.e. sheep red blood cells [SRBC])
RPMI	Roswell Park Memorial Institute
s.c.	subcutaneous
SCID	severe combined immuno-deficiency
SD	standard deviation
TCR	T cell receptor
Tg	transgenic
Th	CD4 T helper cell phenotype (i.e. Th1/Th2)

1.0 Introduction

1.1 A Parable

The American poet John Godfrey Saxe (1816-1887) helped to popularize an ancient parable, originally found in Hindu scripture, which humorously warns against the danger of false conclusions gained by a limited scope of investigation; six blind men conclude that they are in the presence of six different objects as each touches a different part of the same elephant:

> And so these men of Indostan
> Disputed loud and long,
> Each in his own opinion
> Exceeding stiff and strong,
> Though each was partly in the right,
> And all were in the wrong!

In the history of science, few events are summarized so accurately by Saxe's stanza than the polarized debates between the cellularists and humoralists concerning the protective nature of the immune response in the early 1900's.

The synthesis of individual pieces of experimental data, each alone only "partly in the right" is the basis of science. This introduction will briefly discuss the history of the development of current understanding of the general features underlying the regulation of cellular and humoral effector mechanisms employed by the immune system, with emphasis on the development of models of lymphocyte

(especially T cell) activation, and experimental data from the 1960's and 1970's demonstrating that the immune system could respond against an antigen with either a predominantly cellular or humoral phenotype. These latter experiments first raised the notion of a *decision criterion* by which the immune system could select the most effective means of protection when faced by a particular challenge. Subsequent studies during the 1980's and 1990's provided evidence that, in many different situations, the type of immune response generated – either predominantly cell-mediated, or predominantly humoral, could be a critical variable in determining the efficacy of the response.

What influences the adaptive immune system to make this choice between effector mechanisms, which can be so critical to the outcome of an infection? This question, facing immunologists today, also brings to mind Saxe's poem, and I will conclude this discussion, by reviewing current variables and models of cellular/humoral immune response regulation. Preceding this review, I will first present a brief overview of selected major discoveries in immunology, especially relating to the concept of lymphocyte cooperation in the generation of immune responses. In the second major part of the introduction, I will describe the continued refinement of lymphocyte activation models over the past 25 years, in the context of major, more modern discoveries, and finish with a description of and commentary briefly critiquing the most accepted currently proposed models of CD4 T cell activation.

I chose to order the material in the introduction as stated above because I believe that the most powerful approach to interpreting the relationships between the

several individual factors which will be discussed (in 1.10), and their influence on the Th1/Th2 phenotype of an immune response, is by viewing these variables as also influencing events associated with CD4 T cell activation. Stated more succinctly, that the Th1/Th2 polarization of CD4 T cells reflects, in large part, variables influencing their activation. As this discussion will ultimately show, the "elephant" of CD4 T cell Th1/Th2 differentiation is, of course, too large and complicated for any individual, or any single thesis, to describe completely, or in sufficient clarity. However, at the conclusion of the introduction, I will state an hypothesis, based upon a model of CD4 T cell activation, concerning the Th1/Th2 phenotype development of newly activated CD4 T cells, which I believe is at least "partly in the right". This opinion, I hope, will be supported by current and historical references, and by my experimental data.

1.2 The birth of the study of cellular and humoral immunology

1.2.1 Two separate mechanisms of host defense

Humans and pathogens causing disease have co-evolved (Ewald, 2002). Throughout history, close observation and fortunate insights have led to the protection of individual populations against isolated pathogens, for example, the ancient practice of variolation against smallpox, whereby susceptible individuals were inoculated with material obtained from a smallpox vesicle. Later, the scientific method helped to verify the effectiveness of and improved upon these practices, leading to safer methods of protection, adopted by larger populations, for example, the advent of vaccination against smallpox, introduced by Jenner in England in 1798. But while such innovations were no doubt practical, the basic knowledge of a mechanism proper of immunity brought about by these practices is absent until the late 19th century. At the close of the 1870's, work by Koch and Pasteur led to a revolutionary understanding of infectious disease, summarized by the now familiar Koch's Postulates. Armed further with Darwin's *Origin of Species*, first published in 1853, and the concept of *survival of the fittest*, the new scientific discipline of microbiology now begged for creative minds to probe new questions. Chief among these questions, and replete with the potential to change the human condition itself: what defenses does an organism employ to protect itself from infectious agents, and what is the underlying mechanism or basis of this defense? This monumental

investigation led to the volatile scientific birth of the discipline of immunology in the 1880's.

Elie Metchnikoff's name is most often associated with the Phagocytosis Theory, although several researchers at the time, in one form or another, expressed the general observation in several different systems, that certain single-cell organisms and certain blood cells (leukocytes) could *actively* ingest foreign substances (fungi, dyes, red blood cells), and thus, could potentially serve to protect organisms from foreign substances and infection (Tauber and Chemyak, 1991). These findings later led Metchnikoff to the revolutionary proposal at the time, that inflammation (as a prerequisite for phagocytosis) was an active, potentially protective response mediated by the infected organism and not merely a deleterious symptom brought about by infection.

A separate 'school' of immunologists focused not on populations of phagocytic cells as mediators of immunity, but instead investigated the well-established anti-microbial properties of blood. By 1890, investigators led by Emil von Behring demonstrated that cell-free serum from guinea pigs immunized with either tetanus or diptheria could neutralize toxins produced and released by the specific bacteria and, upon transfer to naive animals, could protect against the same bacterial challenge, but not against a different challenge. Similarly, in 1895, Richard Pfeiffer showed that guinea pigs receiving serum from an immune animal could lyse whole bacteria upon challenge (Silverstein, 1984). Unfortunately for the advancement of immunological understanding, there arose an almost immediate hostility between the cellular and humoral investigators; little attempt was made to

formulate a synthesis between the two observed mechanisms of immunity. Metchnikoff himself rejected any duality of cellular and humoral functions in explaining protection, instead insisting that humoral elements, in situations where they were found protective in the absence of overt phagocytosis, were originally produced by stimulated phagocytes. At the turn of the century, Paul Ehrlich published a comprehensive model, the Side-Chain Theory, describing how a humoral response (antibody) could *actively* be mounted against a foreign substance by an infected organism (Ehrlich, 1900). This represents a tremendous divergence from previous humoral investigators, who either offered no model, or one of many based on exhaustion of vital, pathogen-specific humors in the blood. Ehrlich's model, with its exquisite mechanism of antibody specificity, revealed a fatal flaw in Metchnikoff's view of what was by now well-characterized as *adaptive* immunity, which Metchnikoff would never be able to answer satisfactorily: the question of how phagocytic cells could be phagocytic in an antigen-specific manner.

1.2.2 A synthesis

The proposal of mechanisms by which an infected organism could *actively* protect itself by the activation of phagocytes or by the production of antibody, represents a critical first-step for the science of immunology. This new understanding of immunity replaced the rather passive role played by the infected organism that was incorporated into medical doctrine, largely unchanged, since medieval times. A union of the extreme cellular and humoral positions began to take

shape with Almroth Wright's demonstration of enhancement of phagocytosis of bacteria by their prior opsonization with immune serum (Silverstein, 1984). It could be argued that the Nobel Prize awarded jointly to Metchnikoff and Ehrlich in 1908 "in recognition for their work on immunity" marks the end of the tumultuous infancy of immunology as a scientific pursuit. Indeed, some of Ehrlich's comments seem to attest to a degree of integration of cellular and humoral responses, as the Nobel Prize winning humoral theorist stated his position that protection against cancer "is not due to the presence of antibodies, but is determined purely by cellular factors" (Ehrlich, 1909).

However, in the early 20th century, the thought that inflammation, antibody, and complement could all be components of a unified immune system, working in concert, was largely absent with rare exceptions (for example, Wright). In retrospect, the experimental systems employed at the time might not have been sufficiently sensitive or flexible to observe such interconnections, as extreme examples of polarized cellular or humoral responses were cited by scientists of the opposing cellular and humoral extremist positions, both in print and in debate, without much interest in formulating connections between the two. Importantly for this thesis, observations that the immune system could respond to an antigen with either a predominant cellular **or** humoral phenotype were not yet made, again in large part because the two 'camps' worked with such different antigens; cellularists most often employed a challenge of anthrax bacilli injected intraperitonally, while early humoralists most often immunized with bacterial toxins (Silverstein, 2003). Thus, no model existed at this time for a decision criterion controlling whether the

immune system would respond against *any* given antigen with a cellular or humoral response; indeed, the only readout of most *in vivo* experimental systems at the time was survival after treatment.

1.3 Lymphocytes

1.3.1 Early demonstrations of the importance of lymphocytes in adaptive immune responses

Pioneering immunologists did not often question the detailed identity of protective phagocytes, nor the cells/tissues responsible for antibody production. During the 1920's and 1930's, the majority of immunological research centered around serology, in large part due to the practical effectiveness of passive immunization with a variety of commercially produced antisera, especially against diphtheria and tetanus toxins, and the hope that similar vaccines might soon be available for other devastating diseases. Furthermore, experimental methods were not sufficiently developed at this time to investigate even simple properties and functions of immune cells, especially when isolated *in vitro*, while Landsteiner and other serologists were able to investigate the fine specificity of antibodies to slightly different hapten conjugated antigens quantitatively (summarized in Landsteiner, 1945). James Murphy was a rare exception – from 1914 to 1926, he and his associates showed a role for lymphocytes in several immunological phenomena including graft rejection in chick embryos (Murphy, 1914a), tumor resistance in rats

(Murphy and Sturm, 1919), and tuberculosis resistance in mice (Murphy and Elis, 1914). In the latter two experimental systems, Murphy used repeated doses of radiation to cause an extreme lymphopenia, which resulted in a loss of resistance to the particular challenge. Finally, in 1925, Murphy and Sturm showed indirectly that lymphocytes were potentially responsible for the generation of precipitating antibody in rabbits immunized with horse serum, and provided the protective antibody against pneumococcus, again by using large doses of irradiation to destroy lymphocytes and abrogate specific antibody production present in non-irradiated controls (Murphy and Sturm, 1925). Research by other investigators, especially in the field of tuberculosis, including Boquet (1933) and Krause (1925), also implicated cellular mechanisms of defense as critical in disease resistance, although they do not seem to have attributed this 'cellular immunity' to lymphocytes per say (reviewed in Pinner, 1945). Much later, Landsteiner and Chase gave further support to a role for cells in adaptive immunity when they reported the transfer of delayed hypersensitivity against tuburculin antigen with cells from an immunized guinea pig, but not with serum (Landsteiner and Chase, 1942). Later still, Mitchison reported that the ability to "passively transfer transplantation immunity" was not due to elements in the serum but was a property of living lymphoid cells (Mitchison, 1954). However, the immunological community at the time of Murphy's investigations and long afterwards, dominated still by serologists, seems not to have been influenced by this pioneering work for a variety of reasons (Silverstein, 2001).

Building on Murphy's observations, Topley, in 1930, demonstrated that cells transferred from the spleen of an immunized rabbit produced antibody upon

challenge when transferred to a naive animal (Topley, 1930). It was later shown that this ability of spleen cells to produce antibody was abrogated if the cells were heat-killed before transfer (Harris and Harris, 1954). A year later, Coons et al., through developing the technique of flourecent microscopy, provided compelling evidence that plasma cells produced antibody in response to diphtheria toxoid in immunized rabbits (Leduc et al., 1955). However, as most researchers at the time did, this group found no role for the abundant lymphocytes present in the spleen and lymph nodes in either the primary or secondary antibody response.

In 1959, Gowans confirmed that lymphocytes, which he collected from the thoracic duct of rats and labeled with ^{32}P, recirculated through the blood (Gowans, 1959), and could be found again in lymph nodes, Peyer's patches, but not in the thymus (Gowans and Knight, 1964). Gowans also demonstrated that the large numbers of "small lymphocytes" found to recirculate were not the result of division the of other small lymphocytes, as determined by their inability to be detected with tritium-labelled thymidine, suggesting that this cellular population had a relatively long life span (Gowans, 1959). The first conclusive relationship between lymphocytes and an immune response was made by Gowans when, based on the protocols of Medawar's groups' classic tolerance experiments, he injected small lymphocytes, purified from the thoracic duct of parental strain rats, into adult F_1 hosts, causing robust graft versus host (GvH) reaction (Gowans, 1962). Thus, evidence that lymphocytes behaved in a manner indicative of function – that this population of cells was not simply an inert component of the lymphatic system - and

evidence of the immune function of lymphocytes, albeit in a rather unphysiological role (alloresponsiveness), was only obtained in the early 1960's.

1.3.2 The Thymus

The defining of a biological role for the mysterious thymus gland was of critical importance in further uncovering the identity of the cells responsible for immunity, and their cooperative nature in generating protective responses. Both of these discoveries were prerequisites for the development of models regulating the overall cellular/humoral nature of the immune response. Though the thymus was known to be a lymphocyte-producing organ, and Gowans had shown lymphocytes to be immunocompetent cells, immunologists did not believe it to serve any real immunological function – as late as 1963, Medawar wrote that "the presence of lymphocytes in the thymus [is] an evolutionary accident of no very great significance" (Miller, 2002). This prevailing view was based largely on the findings that, in contrast to peripheral lymphocytes, thymocytes could not transfer immunity in appropriate experimental systems, and because removal of the thymus from otherwise healthy *adult* animals did not appreciably affect the generation of immune responses (Miller, 2002).

The first of a series of watershed investigations on the role of the thymus was published by Miller in 1961. Here, he described symptoms including weight loss, lymphocyte deficiency, failure to reject skin grafts, decreased antibody production, and premature death in mice when the thymus was removed neonatally (Miller,

1961). Furthermore, Miller described the restoration of immunologic function of irradiated mice in a thymus-dependent manner (Miller, 1962). Similar experiments showed that while adult rats, when depleted of lymphocytes by thoracic duct cannulation, could no longer mount primary antibody responses to sheep red blood cells (SRBC) or tetanus toxoid, strong antibody responses could be restored by reintroduction of small lymphocytes from syngeneic rats (Gowans et al., 1962). This body of work led to the belief in the early 1960's that the thymus was responsible for the production of relatively homogeneous precursor lymphocytes able to later differentiate in the periphery and then, upon antigen encounter, mediate both cellular and/or humoral immunity (Burnet, 1962).

1.3.3 Two populations of lymphocytes (T cells and B cells)

Avian species, unlike most other vertebrates, contain a primary lymphoid organ in addition to the thymus: the bursa of Fabricius. (Purely out of interest, some marsupials contain two seemingly redundant thymuses). Using testosterone to inhibit bursa development within chick eggs, it was demonstrated that such "hormonally bursectomized" chickens failed completely to produce antibody, both to proteins and bacterial products, but that these birds did reject foreign skin grafts (Warner et al., 1962). This finding seemed to demonstrate that in birds, separate primary lymphoid organs were responsible for the generation of separate leukocyte populations able to produce either antibody (by bursa-derived cells, or B cells) or able to mount graft-versus-host (GvH) reactions (by thymus-derived cells, or T

cells), while in other vertebrates, the thymus gland did both jobs, producing both T and B cells, or their common precursors, able to differentiate in the periphery according to need and further instructional signals (Burnet, 1962).

However, in a landmark series of experiments, it was discovered that irradiated mice receiving mixtures of bone marrow cells and thymocytes for reconstitution produced significantly more antibody upon immunization than did mice reconstituted with either population alone (Claman et al., 1966a and b). Claman's group was unable to determine which population of cells was primarily responsible for giving rise to the effector cell producing antibody, and which population was required for 'help'. Two years later, using sources of "semi-allogenic" bone marrow and thymocytes which could be separately depleted with different specific antibodies, it was demonstrated that the bone marrow cells contained precursors of antibody producing cells (B cells) and that thymus derived cells (T-cells) contained only "antigen-reactive cells" able to help or facilitate optimal SRBC-specific antibody production (Mitchell and Miller, 1968).

Critically, the synergy of T and B cell populations was demonstrated to be antigen-specific. In adoptive transfer experiments, spleen cells taken from an irradiated host reconstituted with thymocytes and immunized with a non-crossreacting RBC could not enhance antigen-dependent antibody production when used to reconstitute a secondary host challenged with SRBC, in comparison to spleen cells from primary hosts immunized with SRBC (Mitchell and Miller, 1967). Furthermore, it was demonstrated that thymocyte proliferation was required to "help" antibody production, indicating that thymocytes were indeed precursor cells,

giving rise, after proliferation, to peripheral 'helper' cells; this was reflected in the superior ability of 'educated thymocytes' - a population of thymocytes obtained after serially passage through two irradiated hosts challenged with antigen – to generate antigen-specific antibody responses compared to the ability of freshly isolated thymocytes (described in Miller, 1999).

The experimental demonstration of separate but interacting populations of lymphocytes was crucial to the further development of immunology. Concurrently, the medical community verified the importance of the thymus and of cooperation in the generation of immune responses in the human immune system by observation and treatment of several "experiments of nature" – such as children lacking a thymus (DiGeorge syndrome) and individuals affected with X-linked agammaglobulinemia (Good, 2002). It now became possible to entertain models of activation of immune responses – more precisely, activation of individual immune cells, and their regulation. Importantly, coinciding at this point in the history of immunology with the demonstration of cellular cooperation in the generation of immune responses is the first burst of papers describing experimental immune deviation. This will be discussed further, in some detail, in the second half of this introduction.

1.4 Development of the Two Signal Model of lymphocyte activation

1.4.1 Preamble

In order to understand new questions and concerns brought to the fore of immunological research by the demonstration of two separate but cooperating populations of leukocytes, it is necessary to briefly discuss the evolution of theories of antibody production. This is largely because the central concept arising out of the refinement of these theories, namely that one immunocompetent cell bears only one receptor specificity, later convincingly demonstrated experimentally, was prerequisite for more refined and encompassing models of lymphocyte activation. In the second part of this introduction, I hope to make the case forcefully that lymphocyte activation, and the regulation of the immune response phenotype (cellular or humoral), seem to be intimately associated events. Therefore, any model of immune phenotype regulation should most likely be intertwined with, and be based upon, a valid model of lymphocyte activation.

1.4.2 Clonal Selection Theory

In 1955, Niels Jerne proposed a model of antibody formation, in many ways reminiscent of Ehrlich's Side-Chain Theory published half a century before, based in large part on the diversity of 'natural' antibodies present in an unimmunized animal, which he had investigated two years earlier (Jerne, 1955). Jerne's Natural Selection

Theory of antibody production was the first to *embrace* (as compared to Ehrlich, for whom it was a monumental concern) the tremendous number of separate antibody specificities needed, as revealed largely by Landsteiner's work, without incorporating a purely instructive mechanism, as was proposed most notably by Haurowitz and later by Pauling in the 1930's and early 1940's. While the Natural Selection Theory did not survive long in the face of increased understanding of the how genetic material encodes protein chains, Jerne's concept of a rather complete, diverse repertoire of natural antibodies would later be embraced by many theories of lymphocyte activation, mostly in recognition of the enormous diversity of antibody molecules.

Talmage, for a time overlooked in the development of the final formulation of Clonal Selection Theory, built upon Jerne's model by hypothesizing that the entity involved in selection by specific antigen was not an antibody molecule, but rather a cell expressing an antibody molecule on its surface. Upon encountering antigen, rare cells bearing the appropriate antigen-specific receptor would proliferate extensively, and thereby produce high titers of specific antibody (Talmage, 1957). Talmage's model was supported by years of histological observations of lymph node and spleen, which documented extensive lymphocyte proliferation in response to immunization. Shortly after Talmage, Burnet published the core of his Clonal Selection Theory (Burnet, 1957). In this brief communication, Burnet incorporates Talmage's refinement of Jerne's proposal, and adds a further restraint that would prove a critical insight into the regulation of immune responses: "the expendable cells of the body can be regarded as belonging to clones ... each such clone will have

some individual characteristic" (Burnet, 1957). Burnet goes on to tentatively define lymphocytes as cells that bear *one specific antibody receptor each* – and that activation of one clone will result in its multiplication and specific antibody production. Shortly afterwards, Burnet elaborated on these core assumptions and as well, offered brief explanations for the difference between primary and secondary antibody responses, self-tolerance (very similar to Jerne's and Talmage's proposals), and affinity maturation (Burnet, 1959).

In 1958, Nossal and Lederberg demonstrated experimentally that in mice immunized with two separate serotypes of *Salmonella*, one lymphocyte, painstakingly isolated from the popliteal lymph node, produced antibody of only one specificity (Nossal and Lederberg, 1958). This landmark result was repeated in a number of similar systems in the following years, primarily because of conflicting results obtained by Cohn and Lennox, showing that about twenty percent of lymphocytes produced more than one antibody specificity (Attardi et al., 1959) – perhaps because single cells had not been as rigorously isolated (Nossal, 2002). Further support for the basic tenets of Clonal Selection Theory was published in 1966 – experiments were published estimating that the number of lymphocytes in a mouse spleen capable of responding to SRBC was about $1x10^3$ (Kennedy et al., 1966) and the demonstration of very rare specific antigen-binding cells in the spleen (Naor and Sulizneau, 1967). Finally, using a panel of fluorochrome-labeled antibodies, it was shown under the microscope that in mice, antigen (polymerized flagellin) bound to a very low percentage of spleen cells in unimmunized animals, and that this binding could be inhibited by pre-exposure of the spleen cells to anti-

immunoglobulin antibodies, that the number of antigen binding cells increased dramatically after immunization with flagellin, and finally, that each antigen-binding B cell expressed one receptor specificity (Raff et al., 1973). These observations, combined with an increasing understanding of genetic regulation, especially the demonstration of allelic exclusion, cemented the central tenets of the Clonal Selection Theory.

1.4.3 Early models of lymphocyte activation

Inherent, though not specifically addressed in the formulation of the core of Clonal Selection Theory, is a 'one-signal' mechanism of lymphocyte activation: a cell bearing a receptor specific for a foreign antigen binds the antigen (signal one) and thereby is activated to multiply and produce antibody. However, even Landsteinder's relatively simple hapten-carrier experiments placed this basic model in doubt. A refined one-signal model arose which dealt with hapten-carrier effect by stating that antibodies recognized both a portion of the carrier and of the hapten (Levine, 1965). This model would prove to be a short-lived, and final reprieve for one signal models of lymphocyte activation.

Theories of self-tolerance have largely guided the development of models of lymphocyte activation, a reminder of the dread specter of Ehrlich's *horror autotoxicus* first raised at the turn of the 20th century. Lederberg had proposed a model of lymphocyte activation, within a self-tolerance framework, in which *the maturity of the cell* at the time of specific-antigen encounter determined whether or

not the cell was activated or inactivated. Immature cells upon interaction with antigen were inactivated, leading to a state of antigen-specific tolerance, while mature cells, following the same interaction, were activated leading to a state of immunity. Central to this model was the concept that the early and continuous presence of antigen was required in order for an antigen to be considered self (by inactivating specific cells at a time when they are inherently tolerizable). This model was most famously supported by the tolerance experiments of Medawar and his colleagues in mice, where *in utero* exposure to foreign cells rendered these mice tolerant to skin grafts at eight weeks after birth (Billingham et al., 1953).

1.4.4 Talmage and Pearlman: one and a half signals

The observations of researchers such as Dresser, who showed that the same antigen (bovine gamma globulin) could induce both antibody production and tolerance when administered in a different manner to adult mice, depending on the dose and form of the antigen (aggregation) (Dresser, 1962a and b), and others who showed a similar pattern in guinea pigs (Battisto and Miller, 1962), combined with an accumulating wealth of hapten-carrier data, led to the formulation of a new model. This model of lymphocyte activation was also based on clonal selection theory (but somewhat loosely, as the authors do entertain that an antibody producing cell may express more than one antibody specificity) (Talmage and Pearlman, 1963).

Two simple but critical observations form the foundation of this proposal. First, the observation that plasma cells producing antibody themselves do not divide

(Perkins et al., 1961), and second, that individual antibody producing cells seemed to produce the same amount of antibody during a primary and secondary response (Uhr et al., 1962). These two observations, when viewed within a selective (rather than an instructive) framework of antibody production, combined with the large increase in cells seen in lymph nodes and spleen during a secondary immune response, suggested that the ability of the *pre*-antibody-producing cell to divide is the most important factor in determining the outcome of a specific antigen-antibody receptor interaction.

Talmage and Pearlman ascribe specificity of antigen for antibody as signal one (as before), but importantly, they introduce a "non-specific" *second signal* (or perhaps, better described as a second variable) controlling whether or not the signal one receiving clone can divide enough to produce a measurable amount of antibody. This additional signal also separates the new model from Lederberg's in that lymphocytes *need not go through an inherently tolerizable state* – the conditions at the time of immunization (controlling the generation of a second signal) determine the outcome of antigen-specific antibody reactivity on a clonal level. Largely influenced by Dresser, the mechanism of activation places control over the delivery of the second signal on the degree of antibody aggregation, and the associated level of complement fixation by the antigen. Specifically, after internalization of the antigen-antibody complex (via surface immunoglobulin), "intracellular complement components may be thought of as inhibitors of cell division, and adsorbtion by antigen-antibody complexes [within the pre-plasma cell] releases the cell from this inhibition" (Talmage and Pearlman, 1963).

This, by extrapolation, placed control of the second signal on the nature of the antigen – fitting Dresser's data nicely– an antigen with many determinants recognized by antibody would result in more aggregation of antibody, whereas an antigen with few determinants recognized by antibody would not result in aggregation beyond a critical threshold. Furthermore, the model encompasses a mechanism of cooperation in the generation of immune responses, "since it is unlikely that natural proteins possess closely spaced identical determinants, the fixation of complement [second signal] probably requires the presence of antibody directed to two or more different determinants" (Talmage and Pearlman, 1963). Additionally, the authors appreciate how the history of an individual, such as a Dresser-tolerized guinea pig, could impact a specific immune response upon challenge, "once tolerance is established, antigen must be maintained to prevent significant accumulation of cells with potential [to cooperate and respond], presumably, from the thymus" (Talmage and Pearlman, 1963). This model then has built into it a mechanism of self/non-self discrimination, addressed by the authors in some detail: a self protein is unlikely to ever induce an immune response as cells specific for a self determinant would be tolerized as they are generated one, or very few at a time, in the absence of cooperation (the absence of aggregated antibody). The mechanism of self-tolerance is dramatically different from the time-centered mechanism proposed in Lederberg's model.

The possible 'priming problem' evoked by this mechanism – how several different antibody specificities could be present before cells could be activated to produce them – was dealt with easily by proposing a low level of "pre-circulating"

antibody to be present in the serum before antigen encounter – Jerne's natural antibody. The authors conclude their manuscript by predicting that (i) peptides derived from an immunogenic protein will be tolerogenic if used as antigen and that (ii) peptides will regain their immunogenicity when given together with an adjuvant. These predictions have been proven correct, although with a significantly different interpretation (for example, Peters, 2003).

The problem that the Talmage and Pearlman model would face, when confronted with the more complex carrier effects described below, is summarized nicely by borrowing a phrase from Dresser, - that the model relies on, "the action of a non-specific substance in determining which of the apparently exclusive states [tolerance or antibody production] an antigen will induce" (Dresser, 1962a).

1.4.5 Carrier effects and their impact on early models of lymphocyte activation

Simple hapten-carrier experiments, largely developed by Landsteiner, had been described since the early 1900's, and led to the critical observation of the almost unlimited range of antibody specificities, which in turn would eventually lead to the birth of Clonal Selection Theory arising out of the instructive/selective debate. More refined studies of hapten-carrier systems, and their conclusive demonstrations of cooperation between leukocytes in the generation of immune responses did not readily fit into the remarkable 'catch-all' single-receptor Talmage and Pearlman model framework that had explained so many immunological phenomena. These

more subtle hapten-carrier results also placed Levine's and related 'local environment models', which suggested that hapten-specific antibody recognized elements of the carrier as well, in serious doubt.

Using hapten conjugates of poly-l-lysine (h-PLL) in complete Freund's adjuvant as antigen, certain "responder" guinea pigs mounted both an antibody and delayed-type hypersensitivity response against h-PLL while other "non-responder" animals could not respond at all to the conjugate antigen (Kantor et al., 1963). The responder/non-responder phenotype was shown to be genetically determined; however it was shown not to be dependent on strain-specific differences in the availability of antigen in secondary lymphoid organs, as injected 2,4-dinitro-phenol (DNP) -PLL products were found to be equally present in the lymph nodes of both responder and non-responder animals (Levine and Benacerraf, 1964). The phenomenon was furthermore shown not to be restricted to the antigen DNP-PLL specifically, as several other haptens responded identically when coupled to PLL (Levine and Benacerraf, 1964).

Furthermore, and critically, non-responder animals were found to be able to produce antibodies, but not delayed-type hypersensitivity, specific for DNP-PLL when complexed with foreign albumin (bovine and ovalbumin) proteins but not when complexed to guinea pig albumin (Green et al., 1966). Delayed-type hypersensitivity was seen in these experiments only against the 'new' carrier albumin proteins. In effect, these experiments treated DNP-PLL as a hapten, conjugating it to an immunogenic carrier. Finally, when tolerance was induced to the carrier albumin in guinea pigs before immunization with DNP-PLL-carrier,

antibody production was abolished (Green et al., 1968). These sets of data made it difficult to accept a model of lymphocyte activation that is centered around the recognition by a specific receptor, of a combined site formed by the hapten and carrier molecules. Furthermore, it was demonstrated that when immunized with the conjugate hapten-carrier antigen, separate anti-hapten and anti-carrier antibodies were synthesized by separate cells (Green et al., 1967).

A similar system, employing lactic dehydrogenase isoenzymes as antigens in rabbits, produced strikingly similar results (reviewed in Rajewsky and Rottlander, 1967). Rajewsky's data related the complex hapten-carrier effects to a whole, intact antigen, thereby demonstrating a probable physiological relevance for the reams of observations on hapten-carrier systems. Clearly, when faced with these new observations, the simple 'one' and 'one and a half' signal models of lymphocyte activation and antibody production, were found wanting.

1.4.6 Two signals – no model

The Cold Spring Harbor Symposium held in 1967 focusing on antibody formation offers a revealing look at the state of lymphocyte activation models at the time. The recent observations of Benacerraf, Rajewsky, and other groups had clearly impacted the immunological community, which now began to realize that, "some element [was] missing from the minimum theory" of antibody production (Mitchison, 1967). The missing step, a still nebulous second signal, is alluded to throughout the proceedings, for example: "suggests that two or more receptors are

involved in a cooperative act of recognition" (Mitchison, 1967), and, "an induction step [in the formation of antibody] which involves a reaction with the carrier molecule" (Benacerraf et al., 1967). In speculating on the nature of the cooperative inductive step (based on the carrier effect), Rajewsky and Rottlaender suggest that "cellular receptors", and not circulating antibody, are responsible that may "correspond to the specificity of certain antibody combining sites, or be entirely unrelated to any antibody specificity" – this 'stab at it' seems a foreshadowing of BCR-bearing - TCR-bearing cell cooperation. However, despite observations on cellular cooperation in the generation of antibody, Mitchison may have spoken for a majority of immunologists at the time (before a clear definition of T and B cells) when he remarked that, "the temptation is to leave in the lap of the macrophage all the troubles which beset the minimum theory" (Mitchison, 1967). Because of the importance of this central immunological unknown, and the elucidation of more discrete cellular sub-populations within the lymphocyte population that was occurring, it is therefore perhaps not surprising that within one year, the basis for a new understanding of immune regulation/activation would be presented and elaborated upon.

Also, in 1968, Forsdyke published a brief hypothesis musing that, much like a liquid scintillation counter's "coincidence circuit", the immune system might only respond to an antigen in a situation when antibody-antigen reactions are sensed above a certain threshold (Forsdyke, 1968). Forsdyke predicted that the only certain differences between self and non-self antigens were likely to be "determinant concentration", that is, the number of determinants on a given antigen recognized by

the antigen-specific receptors (a higher concentration for more foreign antigens) and that self-antigens were likely to be continually present. Like Burnet, Forsdyke imagined a unique period in the antibody producing cell's life, when, shortly after its release (from the thymus in Forsdyke's description), specific binding with "self-determinants likely to be constantly present" was lethal. In comparison to these self-reactive cells, which were eliminated as they were generated, cells with specificities against foreign-determinants collected over time, and by the release of Jerne's natural antibodies, created a "buffer" against which the immune system could sense a change in the concentration of foreign antigen. Specifically, if enough foreign antigen impinged on the immune system to bind all the specific natural antibody, then the excess antigen would be free to bind the antibody-producing cell's surface receptor, resulting in an activation signal, antibody production, and clonal expansion. Though Forsdyke's model is similar to that of Talmage and Pearlman, relying on only one signal (different amounts of antibody binding), it represents a refinement of concepts, and was a forerunner to future models of self-tolerance, which would contain a more sophisticated mechanism of lymphocyte activation.

1.4.7 The Two Signal Model of lymphocyte activation

A minimal model accounting for the well-supported concept of cellular cooperation in antibody production was proposed and elaborated on by Bretscher and Cohn, within the greater context of self-non-self discrimination, much like that of Talmage and Pearlman (Bretscher and Cohn, 1968, Bretscher and Cohn, 1970).

Their model retains 'signal one' as the specific antigen-BCR interaction, and introduces an, eventually, *discrete* second signal, delivered by a "special" antigen-specific carrier antibody, when also complexed with the specific nominal antigen, on a *distinct* second antigenic site. This represents a very large step away from the Talmage and Pearlman model in which the specificity of antigen for a specific antibody receptor acted as a bridge for interpreting a second signal *not at all related to the specificity of the antigen, but to the physical properties of the bound antigen-antibody complex*. However, the general need for cooperation (albeit between only one class of lymphocyte in the the Talmage and Pearlman formulation), the effect of an individual's history prior to immunization, and the mechanism of self/non-self discrimination are quite similar when comparing the two models.

Although in their initial formulations the authors briefly mention the possibility of the carrier-specific antibody as being associated on the surface of a "unispecific cell", they largely dismiss this option, as it incorporates into the model a *scarcity problem*, namely that two "presumably fairly rare" cells would have to interact in the generation of an immune response (Bretscher and Cohn, 1970). This 'scarcity problem' did not stop immunologists at large from entertaining this possibility however, as is demonstrated by Mitchison's (quite prophetic) statement in defense of specific cell interaction: "I do not believe that lymphocytes are a gas. One population moves past the other" (Sterzl and Riha, 1969). Others suggested a role for the antigen presenting macrophage in overcoming the scarcity issue: Weigle - "It may be that the bone marrow and thymus cells are brought into close proximity to each other on the macrophage" (Sterzl and Riha, 1969) – this prediction of a three

cell interaction would prove more relevant in T cell activation, rather than B cell activation, as will be discussed further on.

However, the by then well established role of the thymus is addressed in Bretscher and Cohn's elaboration of their minimal model, as thymocytes are envisaged to give rise to the carrier antibody-producing cell – the antibody interacting with specific antigen separately from the producing cell, but perhaps (the possibility favored by Bretscher) still in a cell-surface dependent manner, perhaps on a macrophage or related cell type, as the "special" carrier antibody was thought to be "cytophilic" (Bretscher and Cohn, 1970; Bretscher, 1972).

The possibility of a simple two signal model based on cytophilic antibody was dealt a severe blow when it was shown that the carrier-specific helper activity in adoptive transfer experiments was confined to thoracic duct lymphocytes from carrier-primed mice, and not macrophages obtained from the peritoneal exudate of primed animals (Mitchison, 1971). Furthermore, there was no difference seen in the immune responses of mice reconstituted with peritoneal cells from naive, immune, or tolerized animals. Finally, though crude by modern standards, macrophages were discounted as helper cells (bearing cytophillic T cell-produced antibody) because of their lack of circulation through the lymphatic system, as compared to lymphocytes, as Nossal stated that he had never "seen one of these 'antigen-picker-up cells' actually physically moving around the body and giving a 'stolen kiss' to the bone-marrow derived cell" (Sterzl and Riha, 1969). These experiments could not rule out conclusively a model in which the cytophilic carrier antibody bound cell surfaces for only a very brief period – implicit in Mitchison's experimental design is the belief

that carrier antibody, once bound, would remain on the macrophage's cell surface for a substantial period of time.

In the first formulation of the model, the second signal - the effect of the carrier-specific antibody - changes the final outcome of the first signal, generated as a consequence of antigen-receptor antibody binding – a sort of signal editing rather than a separate and discrete event: "the signal for antibody induction occurs when receptor antibody is stretched between two or more ... determinants and that ... carrier antibody is needed to ensure that two or more antigen molecules bind ... the receptor" (Bretscher and Cohn, 1968). In the 1970 formulation, the carrier antibody is renamed 'associative' antibody for clarity's sake, and delivers a separate, discernable signal to the cell: "the obligatory associated recognition by way of associative antibody (inductive signal) involves a conformational change in the carrier antibody, leading to a second signal to the antigen-sensitive cell" (Bretscher and Cohn, 1970). Also, this second signal was now recognized by a distinct, separate receptor, a conserved "interaction sensing unit", very similar to the modern co-stimulation paradigm, which will be discussed at a later point in this introduction. A further refinement/re-statement of this model – based on the principle of associative recognition - introduces the possibility that, if the associative antibody, when interacting with antigen, is normally present on the surface of a (undefined) cell for which it is cytophilic, then, "signal two could be mediated by a membrane-membrane interaction" between the B cell and the associative antibody bearing cell, or, "the interaction of associative antibody with antigen could lead to the release of a molecule of short range which mediates signal two" (Bretscher, 1972).

1.4.8 The model's infancy

The Two Signal Model accounted for a vast amount of experimental data, including high and low zone tolerance, self-tolerance and critical aspects of induced immunological unresponsiveness and recovery. Conclusive evidence that the carrier primed 'helper cells', able to influence the antibody responses against haptens, were thymus derived T lymphocytes was obtained using antibody mediated complement lysis of cells expressing the surface marker Thy1, known to identify thymus-derived cells among splenocytes. The elimination of Thy1 positive cells from carrier primed spleen, adoptively transferred into irradiated hosts, eliminated the population's ability to support a secondary hapten-specific response upon challenge with the hapten-carrier conjugate (Raff, 1970).

New single-signal models were also proposed to describe the carrier effect. For example, Mitchison explained the need of cooperation between T and B cells by stating that "helper cells [T cells] pick up antigen via specific receptors which combine with one set of determinants; this increases the effective local concentration of other antigenic determinants in the vicinity of the receptors on AFCP [B cells], which are consequently triggered" (Mitchison, 1971). It is interesting, in hindsight, to note that this type of cooperative 'Antigen Focusing' Model gives to T cells the more passive role of APC, and places the decision to respond or not solely on the B cell, a situation which would eventually be somewhat reversed.

While two signals 'won the day' in terms of immunological dogma – winning more by default originally than by wide acceptance, probing the exact nature of

signal two lacked sufficiently incisive experimental systems. Thus, Metzger's words, fittingly written in a contemporary review of the nature of antigen receptors, proved prophetic: "It is unlikely that any molecular models drawn at this time will prove to be accurate in detail, but they can point the way to productive experimentation" (Metzger, 1970).

While the regulation of the T-cell produced associative antibody is not addressed at great length in the 1968 and 1970 papers, in 1972, Bretscher discusses this aspect of the model, so critical for self-tolerance, in some detail. The conclusion of this discussion is a model in which "T cell and B cell induction [is] very similar" (Bretscher, 1972). This idea is largely formulated out of observations that the second signal mediating factor (associative antibody) could be either paralyzed or induced in much the same manner as B cell antibody responses. I have already discussed experiments in which tolerizing animals to a protein before its use as a carrier abrogated antibody production to the hapten, presumably by eliminating T cells or associative antibody specific for the carrier (Green et al., 1968). It was also shown that while prior immunization with a conjugate X-h resulted in a secondary antibody response to the hapten on re-challenge with X-h, challenge with Y-h resulted in poor antibody production and that a secondary response to Y-h could be generated if animals were first immunized with X-h and Y (Rajewsky, 1969). On the basis of these experimental findings, Bretscher introduces a critical new theater for the two-signal model, that of T-cell activation, when he postulates "that T cells must in general be present for the induction of precursor [T] cells" (Bretscher, 1972). This will be discussed shortly. Briefly though, the lack of any demonstration of a cell-

surface bound T cell receptor, and a lack of sophisticated *in vitro* assays, made any descriptive *molecular* model of direct T cell – T cell cooperation, and thus T cell-dependent T cell activation, quite implausible. The detailed study of lymphocytes in allo-antigen responses *in vitro* would eventually open this possibility fully.

1.5 Interlude

Contemporary to the development of valid models of lymphocyte activation, researchers began to notice the 'heterogeneity' of immune responses which could be generated against a specific antigen. By the late 1960's, a wealth of experimental data quietly accumulated, which suggested than an antigen-specific response could be manipulated to be either predominantly cellular or humoral in nature.

I choose here to follow the evolution of the Two Signal Model to its current form, highlighting briefly the critical insights which have guided its (and immunology's) refinement. Within the context of fully stated, modern models of lymphocyte (specifically CD4 T cell) activation, in the final sections of this introduction, I will review the development of understanding that the immune system's response phenotype towards an antigen can be influenced by several variables. I will then state an experimental hypothesis that attempts to describe the decision criterion by which the immune system chooses between different response phenotypes with which to respond against any given antigen under a given set of circumstances.

1.6 Metzger's prediction: evolution of the Two Signal Model

1.6.1 The antigen bridge

After extensive work on alloreactivity, Lafferty and Cunningham published a brief model, based largely on Bretscher and Cohn's framework, which attempted to unify the mechanism underlying the unique responses generated against alloantigens to that governing responses generated against normal antigens (Lafferty and Cunningham, 1975). In the course of their research, Lafferty's group found that 'antigen' alone was not enough to activate lymphocytes in culture (in terms of proliferation and allo-specific cytotoxicity). First, only certain types of alloantigen-bearing cells could stimulate allogeneic reactions, and second, these special cells could only stimulate responders if metabolically active, as cells killed by a variety of means lost their stimulatory (S^+) phenotype (Lafferty et al., 1978). Clearly then, recognition of antigen alone (on the surface of an allogenic or xenogenic cell) was not enough – a second signal was required, and in contrast to Bretscher and Cohn's associative antibody (or T cell), the second signal in allogenic responses was ascribed to phenotypically distinct stimulator *cells*, "which provide a source of the inductive signal as well as antigen", and, experimentally, these stimulator cells were found to be "cells of the lymphocyte-macrophage class" (Lafferty et al., 1978). Applying a modern perspective, Lafferty is describing a class of antigen presenting cells, capable of activating T cells.

In broadening their model from activation of alloreactivity to lymphocyte activation in general, the authors suggest that "allogenic interactions are an artefactual expression of [the] normal inductive process" – Lafferty et al. again propose that the second signal is delivered by a stimulator cell "to which the responder cell is bound by means of an antigen bridge" (Lafferty and Cunningham, 1975). Thus, succinctly put, "antigen controls the specificity of interaction between stimulator and responder cell" – a substantial refinement to a brief 'guess' made by Mitchison, Rajewsky, and Taylor, which employed the same mechanism, *but two, equal, cooperating cells*: "Antigen serves merely as a link, and that the essential element in triggering the response is the bringing of two lymphocytes together surface to surface" (Sterzl and Riha, 1969). In the 'Antigen Bridge' Model (a term also used by Mitchison et al. in 1969, and Bretscher and Cohn in 1970), this antigen-based specific interaction of responder and stimulator resulted in signal two, later termed *lymphocyte costimulator*, "an inductive stimulus that can cause proliferation and differentiation of the responsive cell" (Lafferty and Cunningham, 1975).

Importantly, the demonstration, in the alloantigen system of an APC interacting with, and activating a T cell made plausible Bretscher's earlier prediction that T cell activation might also be governed by two signals as is B cell activation. However, Lafferty's model alone could in no way explain self tolerance or several other *in vivo* phenomena, in part perhaps because it grew out of observations made *in vitro* using the unique alloantigen system. Specifically, the core statement that, "antigen controls the specificity of interaction between stimulator and responder" leaves many unanswered questions. First, if the stimulator and responder are both

antigen-specific, then the model is no different mechanistically from the Bretscher and Cohn model. However, if the stimulator cell is not antigen-specific, then there appears to be no mechanism of self-non-self discrimination stated in any of Lafferty's proposals, making Lafferty's model physiologically unappealing. Still, with the caveat that the S^+ phenotype can be controlled in an antigen-specific, T cell dependent manner, as predicted by Bretscher, the introduction of a non-B cell APC is a significant development.

1.6.2 Complexities solved add complexity to the model

The discovery of MHC restriction in governing immune responses revolutionized understanding of immune regulation and lymphocyte cooperation. Not surprisingly, the Two Signal Model of lymphocyte activation was soon modified to incorporate these seminal findings. The H-2 antigen, originally described as one determinant by Gorer while studying graft rejection in inbred mice, gradually grew into a number of proteins encoded by a complex cluster of genes, which retained the name H-2 (Silverstein, 1999). A definite link between the ability of inbred mice to respond against specific antigen and the Ir region of the MHC was established at around the time of the first publications outlining the two-signal model (McDevitt et al., 1972); however, the mechanism of the genetic regulation of responsiveness remained unknown. Experiments soon showed a need for MHC compatibility in T cell – B cell cooperation-dependent antibody responses; carrier primed (effector T cell source) and hapten primed (B cell source) spleen cells from strains expressing

different MHC II (either different congenic strains or F1 and parental strains), when adoptively transferred together into an irradiated host and given relevant conjugate antigen, could not generate significant antigen-specific antibody (Katz et al., 1973a and b). An experiment using more purified cellular populations obtained from inbred guinea pigs *in vitro* validated this finding and extended a need for MHC matching between APC and the T cell being activated; cultures of macrophages, pulsed with relevant antigen and treated so as not to divide, were tested for their ability to stimulate the proliferation of antigen primed T cells. These experiments conclusively showed that proliferation of T cell populations only occurred when macrophages and T cells originated from the same strain (Rosenthal and Shevach, 1973). These results were extended to a murine system, with defined MHC genetics (Yano et al., 1977).

A similar need for MHC compatibility was demonstrated in systems employing *cytotoxic* T cell responses. A clear role for obligatory MHC compatibility was demonstrated by Zinkernagel and Dohrety when they showed that Lymphocytic Choriomeningitis Virus (LCMV) -specific cytotoxic cells could only lyse infected target cells *in vitro* if they shared the same haplotype – recognizing "altered self" determinants (Doherty and Zinkernagel, 1975; Zinkernagel and Doherty, 1975). This phenomenon was shown to be more general in other, more complicated systems utilizing hapten-coupled targets (Shearer et al., 1975) and CTL's raised against minor (H-Y) histocompatibility antigens (Gordon et al., 1975). These findings, and the identification of the specific restricting elements (surface antigens) that soon followed (reviewed in Paul, 1984) led to an updated formulation of the two-signal

model that was able to account for evidence of lymphocyte cooperation in the context of MHC restriction.

1.6.3 MHC restriction frees the Two Signal Model

A short editorial introduced to models of lymphocyte activation "a simple, conservative explanation of the H-2 restriction of interactions between lymphocytes" (Cunningham and Lafferty, 1977). The authors postulated that in the process of primary T cell activation, "cells become sensitized not to Ag alone, but to this antigen plus a bit of the surrounding surface of the stimulator cell". The well-defined dependency of K or D matching for cytotoxic responses, and I-region matching for T cell helper function is incorporated into the model by describing these different "presenting structures" as an "area code" that allow interaction of cells responsible for different classes of immunity (which recognize the conserved "class-inducing presenting structures" in addition to specific antigen) (Cunningham and Lafferty, 1977). While the editorial maintains that "T cells carry an immunoglobulin-like receptor", an argument against two receptors on T cells (one recognizing the restricting element, and the other recognizing antigen) is presented (Cunningham and Lafferty, 1977), which would later be proven correct.

Though not stated, the implication of this explanation is that the second, inductive signal, delivered by a stimulator cell, is regulated by the interaction of responder cells with antigen *and* (together with) the restricting element. In terms of mechanism, this model is very near the original Bretscher and Cohn model (1970

formulation) in which a conserved receptor, regulating induction by signal two, the "interaction sensing unit" 'sees' the interaction of antigen with associative antibody (signal two) in addition to the surface antibody-antigen interaction (signal one); one of Cunningham and Lafferty's terms for the restriction element in their explanation is "interaction antigen" – when this conserved element is recognized (signal two) as well as antigen (signal one), activation is induced. Thus, the demonstration of MHC restriction made more plausible, especially for T cell activation, the specific cellular interactions proposed by Lafferty and Cunningham (1975), employing a two signal mechanism based on that first proposed by Bretscher and Cohn (1970).

K and D loci products were later termed class I MHC molecules, consisting of a single polypeptide chain in association with a shorter, conserved polypeptide not encoded for in the H-2 loci (β_2 microglobulin), and the I-A and I-E products termed class II molecules, consisting of two polypeptide chains (Klein, 1979). The I region, in addition to the genes encoding the MHC II molecules was also thought to encode the immune response genes, governing certain antigen-specific responses. This complexity was resolved when Klein suggested that, "Ia genes are Ir genes" and that the class II molecule could be recognized by a T cell together with antigen in much the same manner as demonstrated for class I restriction by Zinkernagel and Doherty (Klein, 1979). In this regard, T and B cell activation were 'reunited' with regard to mechanism (restriction by different MHC elements). Klein also proposed a possible reason behind the extreme MHC polymorphism and need for restriction in his Blind Spot Hypothesis (Klein, 1979).

1.6.4 T cell subsets

Implicit in early thymus-based models of immune function was the idea that a relatively homogeneous population of precursor cells differentiated upon antigen encounter, influenced by variables in the peripheral tissue, to give rise to more specialized effector T cells, able to mediate, for example, either cytotoxic effects, or able to help in antibody production. In the early 1970's, a further immunological complexity was revealed by the demonstration that the T cell population, even in unimmunized animals, was very heterogeneous, based on surface marker analysis, and that this heterogeneity was also reflected in effector function.

T helper cells were originally characterized by Cantor and Boyse to be Ly 1+, and to represent roughly two thirds of peripheral murine T cells (Cantor and Boyse, 1975). This population was found in several experimental systems to "amplify the functional activity of other cells after stimulation by antigen associated with I-region determinants" (Cantor and Boyse, 1975). More specifically, Ly 1+ T helper cells were found to help in the production of antibodies against several thymus-dependent antigens, including SRBC, BSA, HGG, and KLH (reviewed in Cantor and Boyse, 1977). Helper cells were also found to augment Ly 2,3+ cytotoxic responses, especially when killer cells were plated at low densities in mixed lymphocyte reaction (MLR) assays (Cantor and Boyse, 1975). Finally, Ly 1+ cells, and not Ly 2,3+ cells were found necessary to elicit DTH responses against SRBC, offering strong evidence that, in terms of help/regulation "the broad division between humoral immune responses and cellular immune responses does not correspond to

the division of labor among T cell subclasses" (Huber et al., 1976). I will return to this weighty statement further on.

More detailed analysis revealed that Ly 1+ cells proliferated against I region differences, while the Ly 2,3+ cytotoxic killer population, making up 5-10% of peripheral T cells, reacted strongly against K and D differences and not I region differences (Cantor and Boyse, 1975b). These findings gave strong support to models of T and B cell activation involving an antigen-specific stimulator cell (S+ phenotype, though not antigen-presenting as predicted in the Antigen Bridge Model), as Ly 1+ T cells were found to be competent helpers in all facets of specific immunity.

1.6.5 Costimulator(s) found

A very strong candidate for the elusive 'signal two' postulated by two-signal models was described in 1976 – thymocyte stimulating factor (Paetkau et al., 1976; Chen and DiSabato, 1976), later renamed IL-2 in 1979. IL-2, purified from the supernatant of mitogen, concanavalin A (ConA), stimulated spleen cells (from the rat, mouse, and human – thus, IL-2 was characterized as non H-2 restricted) was found in several experimental systems to support growth of Ly 1+ and Ly 2,3+ T cell lines, to cause thymocyte proliferation, to support antibody production by spleen cells from nude mice in the presence of antigen (SRBC), and to enhance cytotoxic activity in MLR assays employing thymocytes or 'nude' spleen cells (reviewed by Watson and Mochizuki, 1980). Further support of IL-2 acting as a 'lymphocyte

costimulator' came with the demonstration that both live and fixed helper and killer T cell lines could bind IL-2, while untreated thymocytes and naive spleen cells could not, unless treated with the mitogen ConA – implying that activated (via signal one), but not naive T cells expressed an induced, specific, IL-2 receptor (Watson and Mochizuki, 1980).

An added complexity in some of these *in vitro* systems was shown when T cells, devoid of adherent cells (presumably macrophages), were unable to produce IL-2 when stimulated with ConA. Subsequently, macrophages, and more specifically the IL-1 they produced, was found in some experiments, to induce proliferation of thymocytes given ConA (Mills, 1976) and supernatant from LPS-stimulated adherent cells could, when added to T cells free of adherent cells, result in IL-2 production in the presence of ConA. These and similar findings suggested a model of T cell activation in which the generation of 'signal two' (IL-2) was itself regulated by an induced macrophage-derived second signal (IL-1) and similar 'lymphokine-laden' models, requiring several cooperative, I-A restricted events (Wagner et al., 1980, Paetkau et al., 1980).

IL-2 represented the ideal "hormone-like molecule" postulated to be the second signal in lymphocyte activation (Lafferty et al., 1980). In some ways, activation models based on IL-2 represented a step backwards - Watson and Mochizuki, concluding their comprehensive review on the effects of IL-2 muse:

> Although it cannot be concluded that IL-2 is the normal stimulus
> That drives pre-T cells to maturity, it opens the possibility that
> The limiting step in the differentiation of pre-T cells to mature
> Effector T cells may be solely the presence or absence of a

Proliferative signal (Watson and Mochizuki, 1980)

This sounds almost identical to the reasoning of Talmage and Pearlman in their model of B cell activation proposed fifteen years earlier. Furthermore, the well-demonstrated non-specific, soluble nature of IL-2 made it difficult for any model to ensure specificity – and more critically, self-tolerance, a problem first discussed by Bretscher and Cohn in 1968 in the original formulation of the Two Step Model.

1.6.6 Costimulator lost - then found again

Simplicity of thought (Occam's Razor) and cold logic often fail when applied to complex biological systems – see Cunningham's essay pushing 'Gestalt Immunology' (Cunningham, 1978) - as exemplified by the 'die hard' credo of many immunologists investigating T cell – B cell interactions during the 1970's; these words are J.F.A.P. Miller's: "simplicity and logic dictate that the T-cell receptor for antigen should be similar to the surface immunoglobulin of B cells" (Miller, 1977). The long awaited isolation and characterization of the TCR, both □□ and □□, in the mid 1980's, and the discovery of the biological function of MHC I and II molecules to present antigenic peptides to properly restricted TCRs, revolutionized immunological understanding. Lanzavecchia punctuated this period of discovery with his description of antigen-specific interaction between a B cell, recognizing and internalizing antigen via its BCR, and a CD4 T cell, seeing peptide derived from the nominal antigen in the context of the B cell's MHC II (Lanzavecchia, 1985).

While new cytokines continued to be isolated and characterized, and the mystery of the TCR and MHC continued to be resolved, a new branch of research into T cell activation began to describe data supporting predictions made more than fifteen years earlier. Using defined immunogenic peptides of pigeon cytochrome c (PCC) to decorate splenocytes (APC), and then treating these cells with a cross-linker, it was shown that such cells, when injected into a syngeneic host, rendered recipient mice unable to respond (unresponsive in terms of T cell proliferation) when challenged with the same antigen in CFA four days later (Jenkins and Schwartz, 1987; Jenkins et al., 1987). Similar findings were described *in vitro* using long-term PCC-specific T cell clones, which, though previously activated, could assume an experimentally defined 'resting' phenotype. Importantly, in these experimental systems, exogenously added IL-2 and/or IL-1 could not reverse the unresponsive state. Thus, in contrast to some simple models of the action of costimulation in the activation of T cells, exposure to IL-2, in association with relevant antigen seen in the context of MHC II, was in some situations not sufficient for the activation of resting/naive T cells. Interestingly, it was shown that while fixed, antigen-bearing resting B cells and macrophages could not induce the proliferation of specific T cell clones *in vitro*, and instead induced the unresponsive state, previously activated B cells (Con A) and macrophages (IFN-γ treated) given antigen and then fixed could induce vigorous T cell proliferation (Weaver et al., 1988).

These observations suggested that the *critical* costimulatory signal needed to activate T cell proliferation, superceding or synergizing with IL-2's effects, *was an induced cell-surface costimulatory molecule*. This requirement fit in more nicely

with the original two-signal theory. A then contemporary formulation of Bretscher and Cohn's two-signal model stated that the critical costimulatory signal's expression is upregulated on an APC after the recognition of MHC-restricted antigen by a specific TCR (resulting in an APC activation signal), and that the APC is thus able to deliver 'signal two' to the T cell. Upon receiving 'signal two', the now activated T cell was induced to proliferate, largely via clonal IL-2 production (Mueller et al., 1989). Retained from the original Bretscher and Cohn model is that signal one alone (following the recognition of MHC restricted antigen) led to inactivation (as shown by the well-documented unresponsive state).

A legitimate costimulator (receptor) molecule, this time a cell surface associated molecule, was found again with the characterization of CD28 (originally Tp44 in the human), expressed on over 95% of human CD4 T cells and about 50% of CD8 T cells. Monoclonal antibodies directed against this antigen on human T cells, in the presence of anti-CD3 or similar stimulation were found to result in T cell proliferation, IL-2 secretion, and up-regulation of the IL-2 receptor (June et al., 1990). The description of a natural ligand for CD28, the B cell activation antigen B7, transiently expressed on activated B cells and macrophages, completed a plausible costimulatory circuit, restricted largely to activated APC and T cells (Linsley et al., 1990). Functional evidence of B7-CD28 stimulation was demonstrated by the ability of B7 transfected CHO cells to stimulate proliferation of and IL-2 production by CD3-treated CD28+ human T cells – effects completely inhibited by the blocking of CD28 with monoclonal antibodies (Gimmi et al., 1991). Similar observations were made with murine CD4 (Harding et al., 1992) and CD8 T cells (Harding and Allison, 1993). Intracellularly, the signaling pathways stimulated

by CD28-B7 ligation were found to be distinct from those stimulated by TCR-CD3 engagement (June et al., 1990).

In studies with CD4 T cell clones, it was shown that the unresponsiveness induced by stimulating CD4 T cells with non-costimulatory APC (peptide pulsed and fixed) could be rescued by the addition of allogeneic APC (B cells and macrophages) (Jenkins et al., 1988). These results suggested the possibility of two physically-dissociated activation events for T cells (antigen presentation and costimulation); such a possibility violated the strict principle of "associative recognition" proposed by the Two Signal Model as a means of lymphocyte activation in the context of self-non-self descrimination. Mechanistically, a requirement for delivery of signal one and signal two by the same APC (B cell) was demonstrated by showing that mixed populations of B cells, which could either only '*present antigen*' (actually, the stimulation of CD3 via expression of CD3-specific surface immunoglobulin) or only offer costimulation (LPS activated cells), could not stimulate the proliferation of normal, naive CD4 cells whereas B cells capable of both functions could (Liu and Janeway, 1992). These results, from an experimental system employing naive CD4 T cells, not resting clones, demonstrated that the B7-CD28 costimulatory pair fulfilled the major requirements for the putative 'signal two' of T cell activation models in that B7 was not expressed on resting APC, B7-CD28 engagement required cell-cell contact, and the signal was distinct, both on the cell surface and in terms of intracellular signalling, from the MHC-antigen 'signal one'.

1.6.7 Costimulators regulated by costimulators

A further level of complexity in the regulation of T cell activation was introduced with the characterization of the CD40 molecule, found on the surface of B cells (but not on fully differentiated plasma cells), dendritic cells, macrophages,

and thymic epithelium, and its ligand, gp39, later termed CD40-L, expressed on the surface of (anti-CD3 treated) activated T helper cells. Several experiments showed that CD40-CD40-L interaction was critical for T cell-dependent activation of B cells in terms of proliferation and antibody production, both *in vivo* and *in vitro*, in mice and human systems (Noelle et al., 1992; Hollenbaugh et al., 1992). Furthermore, anti CD3-treated T cells or PCC activated PCC-specific transgenic CD4 T cells were found to upregulate CD40-L in a B7-independent manner, and blocking of CD40-L on these T cells, via monoclonal antibody, caused a significant decrease in B7 expression on B cells interacting with antigen-primed T cells, compared to controls (Durie et al., 1994). Also, stimulation of resting B cells with various forms of CD40-L, and usually a variety of cytokines, was found (beyond the activation and antibody production already described) to protect B cells from apoptotic death (as compared to other forms of B cell activation including LPS treatment) and foster germinal center formation (Craxton et al., 2000; Merino et al., 2003).

Thus, a vision of a complex interplay of T cell-APC cooperation in the activation of both cell populations developed. Critically important for models of CD4 T cell activation: "since the level of B7 on resting B cells is inadequate for reciprocal T cell activation, *the signals that regulate the expression of B7 indirectly regulate T-cell activation* by controlling the APC function of B cells" (Durie et al., 1994 – my italics). This statement, if one extends it beyond B7 and B cells - to the growing number of costimulatory molecules found to be important in T cell activation and differentiation - embodies the major point of contention between two-signal based models of CD4 T cell activation which would arise: the means of regulation of costimulatory molecules on APC, necessary to activate naive CD4 T cells. In fact, some seem so befuddled by the complexity of this regulation that they see, "a gulf between our experimential [sic] understanding of T cell activation and

one of our cherished theoretical paradigms, the two-signal model" (Deenick et al., 2003).

1.7 Today's Two Signal Models

1.7.1 Infectious Non-self

To open the 1989 Cold Spring Harbor Symposia on immunological recognition, Janeway introduced a new model of self/non-self discrimination. Janeway's proposal centered around what he termed the "Landsteinerian Fallacy" – the popular conclusion drawn from the hapten-carrier work summarized by Landsteiner some half century earlier that, in the words of Janeway, "all foreign macromolecules are equally able to give rise to an immune response ... that the immune response shows no special predilection to respond to infectious agents" (Janeway, 1989). This conclusion is invalid, says Janeway, because of "the immunologist's Dirty Little Secret" – the near ubiquitous use of (usually microbial) adjuvants to "obtain readily detectable responses to these antigens" (Janeway, 1989).

The model is developed to its revolutionary conclusion, "that the usual means of inducing costimulatory activity [signal two] is exposure of antigen presenting cells to bacteria and would appear to mean that infection is normally required to elicit an immune response" (Janeway, 1989). This statement is indeed revolutionary: as Bretscher pointed out in 1975, discussing the basis of the original two signal model's 'historical' mechanism of self-nonself discrimination, "No other property of either self or foreign antigens has been proposed that can realistically form the basis of a theory of a self-nonself discrimination" than the early and continuous presence of self antigen (Bretscher, 1975). Janeway's method of self-nonself discrimination is centered around evolutionarily conserved "pattern recognition receptors", which,

when triggered by the presence of microbial (infectious) structures, or "pathogen-associated molecular patterns" (PAMPs) (Janeway and Medzhitov, 2002) would induce the expression of costimulatory molecules (signal two), thus initiating immune responses. These pattern recognition receptors are comprised of several different types of conserved molecules, most notably the toll-like receptor family, but also including liver-produced secreted molecules, intracellular, cytosolic receptors, and other cell surface associated receptors (reviewed in Janeway and Medzhitov, 2002). Interestingly, this original formulation of the Non-Infectious Self /Infectious-Nonself Model describes a relatively minor role for dendritic cells as a response-inducing APC, reserving their services for inducing viral immunity, in part because they were found to take up and process antigen "extremely inefficiently". But, in further descriptions of his model, Janeway reverses this opinion, calling DC's the "most important" APC (Janeway and Medzhitov, 2002). While Janeway's insights were indeed revolutionary, while perhaps not completely original, as a similar view on the initiation of immune responses was briefly put to paper by Smith in 1961 (see Peters, 2003), his proposal, it seems to me, includes a fallacy of its own.

1.7.2 A Janewayian fallacy?

Janeway states that the "immune system responds to 'relevant non-self' in the form of infectious agents, but not to innocuous proteins". Countless pieces of evidence support the conclusion that individual proteins (HEL,OVA ...), when given soluble, cannot elicit a strong immune response. It is doubtless true that the immune system did not evolve to respond against these experimental challenges; however, the finding that one can generate a response against a protein, when given with CFA, may not mean that the immune system can *only* mount a response in the presence of

microbial antigen. For example, aggregating protein antigen can make the same protein immunogenic, in the absence of any detectable microbial products (Peters, 2003). Historically, adjuvants have been employed by immunologists to make their experimental systems work.

In the quotation at the beginning of this section, Janeway contrasts "infectious agents" to "innocuous proteins". These are apples and oranges not just because the former contains microbial structures, which are recognized by pattern recognition receptors, but also because of their size, especially their 'immunological size' – by that I mean the number of foreign sites they contain. A bacterium or viral particle is made up of (in addition to other things) many proteins, compared to a single "innocuous protein". Thus, in the absence of any evasion mechanisms, an "infectious agent" is able to provide a much larger set of foreign peptides for immune recognition than is an experimentally administered dose of a single protein, usually derived from a closely related (evolutionarily) animal. By increasing the diversity of the set of foreign peptides, for example, immunizing with an XRBC, or by increasing the number of the same set of antigenic peptides, for example by aggregation, it is possible to generate immune responses to non infectious antigens.

Another way to view Landsteiner's work, in contrast to Janeway's interpretation that it showed "the immune system has evolved to recognize equally all non-self substances", is to see hapten-carrier systems as the first demonstration of the adaptive immune system's capability to recognize, equally, a tremendous range of antigens, when immunized with an appropriate challenge. In this system, adjuvants (microbial products), in addition to carrier proteins, were needed to obtain the data necessary for this conclusion. But to set down the general rule that adjuvants are required for the generation of immune responses is perhaps dangerous, as pointed out in Bretscher's argument, addressing a different observation:

> Should the medium of an in vitro system be deficient in some way,
> By, for example, being too acidic, it would be possible to show a
> Requirement for both antigen and alkali to obtain a response;
> Such observations obviously do not constitute evidence for a
> Two signal model" (Bretscher, 1975)

Landsteiner, and other pioneering immunologists, employed an antigen in their experimental systems that could not generate detectable immune response without its administration in adjuvant. However, as summarized by the quotation from Bretscher, and briefly touched upon throughout this section, this might not necessarily mean that microbial products are an absolute requirement for the generation of an immune response.

Janeway's insights into the importance of innate receptors and defense mechanisms, and their interconnectivity with the adaptive immune system were substantial: microbial products can profoundly influence the course of an immune response, including cellular/humoral phenotypic shaping, as will be discussed shortly. However, a second class of substances was also found experimentally to have a strong effect on cells of the innate immune system, sometimes acting through the same conserved receptors.

1.7.3 A 'Dangerous' two signal model

Polly Matzinger, stimulated but unsatisfied with Janeway's infectious-nonself model, proposed a new idea. In 1994, the Danger Model was proposed, which, on the intellectual surface, is very similar to Janeway's idea (Matzinger, 1994). In Matzinger's framework, infectious-nonself are replaced with "danger" signals, later "danger/alarm" signals (Matzinger, 2002), which, when recognized by evolutionarily conserved receptors expressed by professional APC (dendritic cells), would result in the stimulated APC's up-regulation of costimulatory signal two. The originally nebulous 'danger' signals proposed in 1994 now, after extensive research, have some

identities – for example, heat shock proteins (Melcher et al., 1998), and uric acid (Shi et al., 2003). Another class of 'danger' signal seems to 'crossreact' with Janeway's infectious-nonself receptors. For example, toll-like receptor 4 can recognize both certain bacterial LPS structures as well as heat shock protein 70 (Matzinger, 2002). Here Matzinger's and Janeway's ideas fork dramatically as Matzinger maintains that pathogens have evolved to bind a pre-existing danger receptor (for example, viral attachment), while Janeway's interpretation is that conserved pattern recognition receptors evolved to recognize pre-existing pathogens.

Whatever the case, these two models of CD4 T cell activation are quite similar on a fundamental level, as each changes the core Two Signal Model, upon which each is based, by the addition of a separate 'third' signal, generated and regulated in *an antigen-independent manner*, which in turn controls the delivery of a costimulatory second signal. Both models also place professional APC, namely dendritic cells, in a position of prime importance in controlling the activation of naive T cells. Again, because CD4 T cell activation and Th1/Th2 polarization are by necessity closely linked events, many Th1/Th2 differentiation models, when viewed through these 'third' signal models, literally revolve around activated, antigen-presenting dendritic cells.

1.7.4 Dendritic cells

The emergence of dendritic cells as the professional APC population *par excellence* has, in part, caused a major paradigm shift in immunological research. First described in detail in the mid 1970's, DC were found to constitute roughly one percent of the nucleated cells in the spleen and were found to have a unique functional phenotype, which included poor antigen uptake (Steinman and Cohn, 1973). Experimental evidence for an important biological role for DC, especially

with regards to T cell activation, grew gradually from initial reports that DC, even when present in very small numbers, were far more potent stimulators of allo-antigen responses than other, classical, MHC II bearing APC, namely B cells and macrophages (Steinman et al., 1983). Further experiments found that primary antigen-specific responses *in vitro* and *in vivo* were also induced very well by DC pulsed with relevant MHC II restricted peptide antigen, while similar populations of spleen cells or peritoneal cells failed to induce responses (Inaba et al., 1990). While this report's readout of T cell activation was proliferation, experiments further revealed that injection of antigen loaded DCs (myoglobin) could cause antigen-specific IgG antibody production *in vivo*, after secondary challenge (5 days after DC injection) with soluble antigen. These results strongly suggest a CD4 T cell dependent response, implicating the pulsed DC's role in functional CD4 T cell activation (Sornasse et. al., 1992). These and numerous other studies solidified the role of DC as 'nature's adjuvant', and began to place them in a position of importance for T cell activation, previously largely reserved for macrophages. Evidence supporting a role for DCs in determining the Th1/Th2 phenotype of newly activated CD4 T cells will be discussed further on (see 1.11.2)

1.7.5 The steady state

Steinman's research on the dendritic cell has culminated in his proposal that these cells (or at least certain dendritic cell subsets) are the critical cell type involved in both CD4 T cell activation and tolerance induction. In his Steady State Model,

Steinman acknowledges, in contrast to Matzinger's sometimes aggressive dismissals (Matzinger, 2002), that the presence of autoreactive CD4 T cell clones in the periphery is very real, and could represent a potentially disastrous autoreactive population if activated (Steinman and Nussenzweig, 2002). The Steady State Model's approach to dealing with this threat is, in a way, similar to Bretscher and Cohn's original Two Signal proposal in that the interaction of a naive CD4 T cell with antigen alone, as presented by an 'unarmed' dendritic cell, should lead to the CD4 T cell's inactivation. If the same naive CD4 T cell encounters the same antigen on the surface of an armed dendritic cell, - armed by virtue of the dendritic cell's upregulation of costimulatory second signal, then CD4 T cell activation ensues. The regulation of signal two in Steinman's model, however, shares more in common with the models of Janeway and Matzinger, already briefly discussed in the previous two subsections.

While Steinman agrees that, upon receiving maturation stimuli, mediated by either pathogen-derived signals, 'danger' stimuli, or T cell help (CD40-L), dendritic cells upregulate surface expression of MHC-peptide complexes, costimulatory molecules, and initiate cytokine production, thereby becoming capable of initiating immune responses (Lafferty and Cunningham's S^+ phenotype), he states that such, "maturation creates problems with respect to self/nonself discrimination" (Steinman and Nussenzweig, 2002). This scenario is 'nipped in the bud' by giving dendritic cells the authority to tolerize T cells, which come into contact with their relevant antigen on the surface of dendritic cells during the 'steady state' – periods of (relative) calm, marked by the absence of overt maturation signals. Thus, in the

steady state, dendritic cells in the peripheral tissue, much like dendritic cells present in the thymus, assume the critical task of "defining self in the periphery", thereby avoiding the threat of the "horror autotoxicus" first proposed by Ehrlich (Steinman and Nussenzweig, 2002).

One potential problem with this model is that dendritic cells – immature dendritic cells, while having the ability to take up soluble and particulate antigen, present it poorly to T cells, in part because of low surface MHC expression levels, though the immature DC can efficiently process antigen and load *intracellular* MHC molecules (Banchereau et al., 2002). Dendritic cells would therefore seem to have a relatively poor ability to communicate with T cells at all, before receiving a maturation signal. However, *in vivo* evidence supports a tolerizing role for steady state dendritic cells, as dendritic cell targeted antigen (HEL attached to DEC 205 specific antibody: DEC 205 is a receptor expressed on the surface of some DC) caused the eventual deletion of TCR transgenic CD4 T cells specific for HEL, unless a dendritic cell activation signal was given (Hawiger et al., 2001).

1.7.6 The Two Step Two Signal Model

In the experiment described above, immature, antigen-loaded dendritic cells brought about antigen-specific tolerance by first inducing vigorous proliferation of HEL-specific T cells, which then disappeared at around seven days after immunization (Hawiger et al., 2001). Similar results have been reported in other systems employing transgenic cells (Kearney et al., 1994, Pape et al., 1998). This

type of behavior, seen by antigen-specific clones in the absence of required costimulation, has been incorporated into a new, modern formulation of the original Two Signal Model (Bretscher, 1999). I say 'modern formulation of the original (1968 and 1970) two signal model' because in these predecessors (when applied to CD4 T cell activation), signal two, originating from a helper cell, acted on a precursor helper cell *directly*. The Two Step Two Signal Model, proposed by Bretscher, put this mechanism of 'help for help' in a modern context – that is, signal two is mediated by an APC (the stimulator cell in Lafferty's models). However, in strict opposition to the 'third' signal models briefly introduced above (requiring a *third* signal, delivered by the recognition of danger or of a microbial product, in addition to antigen and costimulation), Bretscher's new model retained the need for a costimulatory signal provided by an effector T helper cell specific for the same nominal antigen, and not 'danger/alarm/pathogen signals', in activating new antigen-specific helpers.

The T helper cell's signal, as mentioned, is not delivered to a precursor T cell directly, but is instead delivered to an APC, thus regulating the APC's S^+ phenotype, which in turn will 'deliver the message' to an antigen-specific precursor T cell recognizing a peptide-MHC complex on the surface of the 'activated' APC, via signal two. In contrast to the simplified 'third' signal models (the third signal being a microbial product or other 'danger' signal), Bretscher's new model substantiates the following statement, as will be described below: "the initiation of T-cell immunity is rather demanding" (Banchereau and Steinman, 1998) – the *regulation* of T-cell immunity is rather demanding for 'third' signal models, as will be discussed.

Step one of Bretscher's model is in many ways no different than the basic 'third' signal mechanism of T cell activation. Precursor T helper cells, recognizing antigen on macrophages or somehow-matured dendritic cells (Bretscher does not specify the mechanism of maturation, but in theory, any third signal, such as those proposed in Janeway's or Matzinger's model is integrateble as regulating the second signal of step one of the Two Step Two Signal Model), receive a costimulatory signal, which induces proliferation (Bretscher, 1999). This proliferative spurt of step one-primed T cells is satisfying, both intellectually and physiologically, because it negates the concern, which has consistently hounded the original two signal model, that the interaction of two very rare, antigen-specific lymphocytes (three in the new model – see below) is unlikely. Cells completing only step one will be inactivated – as seen in the experiment which was introduced at the beginning of this section involving HEL-conjugated to DEC 205-specific antibodies.

Step one-primed CD4 T cells, in order to become fully functional effectors, must pass through a second step, receiving a second costimulatory signal (step two signal two), the induction of which, on the surface of an antigen-specific B cell, is regulated by the delivery of an APC-activation signal (S+ phenotype regulating), delivered by a pre-existing CD4 T cell, dependent upon antigen recognition on the same B cell. Two points. First, the requisite of an antigen-specific B cell for the step two, signal two delivering APC focuses the cooperation of step one primed cells and pre-existing effectors as, "the anti-Q B cell is postulated to endocytose the antigen Q efficiently but not antigens to which the receptors cannot bind" (Bretscher, 1999). This statement is supported by experimental data, finding that the selective

expression in B cells of H2-O, involved in MHC II peptide loading, favors MHC II presentation of BCR-ingested antigens (Brocke et al., 2003; Bryant and Ploegh, 2004). This involvement of an antigen-specific B cell offers a level of security against an effector CD4 T cell, recognizing its specific peptide, helping in the activation of a precursor CD4 T cell specific for a peptide derived from a separate nominal antigen. This situation could conceivably arise often if only a professional APC, such as a dendritic cell or macrophage, able to ingest nominal antigens non-specifically by phagocytosis, were involved in T cell activation. Second, the Two Step Two Signal Model can potentially incorporate 'third' signal as influencing the pace of some of the initial events in the generation of specific immune responses (by influencing step one-associated interactions) – this makes some experimental systems difficult to critically analyze. Thus, the pattern recognition receptors and strong costimulatory capability of dendritic cells can serve a critical role in T cell activation, but the Two Step Two Signal Model proposes that the final outcome of dendritic cell – naive CD4 T cell interactions (step one) is regulated by further cellular interactions (step two).

1.7.7 Evidence in support of the Two Step Two Signal Model: a role for effector CD4 T cells in the generation of primary immune responses

Because of the relative difficulty in assessing the role of pre-existing effector T helper cells inherent in any experimental design (as will be discussed later), and

because of the current wave of research focusing on models of T cell activation not requiring T cell cooperation, direct (versus somewhat circumstantial) evidence necessitating T cell help in the generation of CD4 T cell effectors is limiting. However, strong experimental evidence does exist supporting a critical role for pre-existing CD4 T cells, allowing a non-apologetic discussion. Here I briefly state evidence in support of a role for effector CD4 T cells in the activation of naive CD4 T cells, as this aspect of the two step, two signal model is of central importance to my thesis work.

Bretscher showed that the *in vitro*, antigen-induced generation of effector functions associated with CD4(+) T cells (DTH) from unprimed spleen cells was cell density dependent (Bretscher, 1986*)*. Furthermore, spleen cells cultured at low densities, which did not result in the generation of cells able to mount detectable DTH responses against immunizing antigen (burro RBC and TNP), could be rescued via the addition of a few irradiated spleen cells from a high density culture which could induce DTH. The critical cell in these addition-experiments was found to be a T cell via anti-Thy1 depletion of the transferred high density, DTH-rescuing cell. This result strongly supports a view that more is needed for the generation of a specific immune response than relevant antigen, viable pTh cells, and sufficient APC, and that the 'more' needed can be provided in full by a population of radio-resistant T cells (Tucker and Bretscher, 1982). Importantly, APC and antigen are shown not to be limiting in this system as the addition of a few T cells from a high-density culture, while rescuing the response, presumably through some type of T cell cooperation, did not alter the number of APC dramatically.

A similar type of observation was seen, and has recently been reproduced and expanded using an *in vitro* alloantigen system (Pilarski, 1977; Strutt, 2005). These systems rely on thymocytes as a population of T cells containing only naive competent T cells among those responding to antigen, and has been employed to show that thymocytes are unable to mount effective CTL responses or produce CD4 T cell derived antigen-specific cytokines (Strutt, 2005) against different populations of irradiated allogeneic stimulator cells in culture, even at high responder densities. Responses of cultured thymocytes could be rescued via the addition of a relatively low number of syngeneic spleen cells into the responding cultures, specifically, CD4+ T cells with an activated phenotype ($CD44^{high}$, $CD62^{low}$, $CD45RB^{lo}$). These additional cells were irradiated and so could not give rise *de novo* to activated T cells. This result directly supports the need for a pre-existing CD4 T cell to initiate a primary response by a naive population of responding cells in a system that, while certainly open to some question at the physiological level because of the system's *in vitro* nature, must be considered non-limiting in terms of antigen presentation and the antigen-specific precursor frequency.

The findings discussed above are based on experimental systems in which *in vitro* cultures, specifically arranged so as not to respond to relevant antigen, can be supplemented with an additional cellular population that rescues the response. It is difficult to reproduce this situation *in vivo* because, in a normal, unimmunized mouse, effector CD4 T cells can readily be detected, and the density (if one can still call it that) of responding T cells can only be manipulated with severe measures (depleting antibody, thymectomy, radiation, etc.) that might easily have other,

unintended effects. Thus, a test for the requirement of CD4 T cell – CD4 T cell in the generation of antigen-specific responses *in vivo* requires a more subtle approach.

1.7.8 The *dynamic* Two Step Two Signal Model

Peters and Bretscher used the well-characterized protein antigen HEL, and the strain-specific immunodominant and subdominant MHC II restricted peptides derived from it, to see if the prior removal of peptide specific CD4+ T cell populations affected the response against the whole protein (Peters, 2003). CD4 T cells specific for the immunodominant peptide were inactivated by administering soluble peptide, without adjuvant, in a manner that is known to physically eliminate or anergize specific CD4 T cells (Pape et al., 1998). In such mice, made tolerant to the immunodominant peptide, a challenge of aggregated HEL protein, even with alum, a non-microbial adjuvant, - normally immunogenic, resulted in a greatly diminished response to other HEL peptides. This was not because variables of immunization (dose, route) had changed, or because cells specific for *other* HEL peptides did not exist in these mice, but, most likely, because their activation was not induced. This is difficult to explain with any model of T cell activation that does not place some stress on T cell cooperation. Using this experimental system as a stage for discussion provides an opportunity to perhaps more clearly describe the envisaged roles of effector CD4 T cells, precursor CD4 T cells, and other T cells in transition in the Two Step Two Signal Model.

Importantly, the anti-HEL response seen in non-tolerized mice need not require a pre-existing eTh specific for the immunodominant peptide of HEL to be present in naive mice, which might or might not be tolerized by the experimental regime. Instead, might rely on an initial effector CD4 T cell specific for a cross-reacting antigen. This crossreacting cell might then *initiate* the response, which quickly becomes centered on 'immunodominant' peptide-specific CD4 T cells being activated due to the relative prevalence of such peptide-specific CD4 T cells. This surge of initially activated HEL-specific cells might then be able to cause the activation of precursor cells specific for relatively rare HEL epitopes, or conversely, the activation of relatively rare CD4 T cells specific for other, more abundant, HEL-derived peptides. When precursor CD4 T cells specific for the immunodominant HEL peptide are tolerized, HEL challenge could *still* result in the initial stimulation of the crossreacting effector T helper cells to 'try to help'; however, because of the tremendous decrease in the initial surge of newly activated cells (specific for the immunodominant peptide), the response is unsustainable and peters out. While this is perhaps a baroque explanation of the results, models of CD4 T cell activation that do not incorporate a role for cooperation cannot explain this result easily – for example, it is difficult to imagine any substantial changes in the dendritic cell population brought about by the peptide tolerance procedure, or to imagine the generation of peptide-specific regulatory T cells, facilitated by pre-treatment of naive mice with specific HEL peptides, that down-regulate the response of CD4 T cells specific for other HEL peptides.

I believe that this kinetic formulation of the Two Step Two Signal Model can help make it plausible when applied to 'real' situations, as will be necessary to consider in the proposal of a model of Th1/Th2 CD4 T cell differentiation - the Threshold Hypothesis, based upon the Two Step Two Signal Model of CD4 T cell activation.

1.8 Immune Deviation

1.8.1 Preamble

The realization, that the immune system could respond against an antigen with either a predominantly cell-mediated or predominantly humoral response developed slowly during the 1960's and 1970's. This direction of investigation was originally spurred by experiments, conducted by several groups in both mice and guinea pigs, showing differences in the antibody isotypes generated against a variety of antigens administered in incomplete (predominantly IgG1) and complete Freund's adjuvant (IgG1 and IgG2) (Reviewed in Dresser and Mitchison, 1968). The physiological relevance of the immune system's (experimentally induced) ability to respond against antigen in a polarized humoral or cell-mediated manner was speculated upon during this period, and the first models of phenotype regulation were proposed.

The discovery of distinct cytokine-secretion profiles amongst murine, and later, human, CD4 T cell clones, led to the development of a new paradigm explaining the tendency towards exclusivity of cellular and humoral immune responses, controlled by polarized CD4 T cell subsets. This paradigm has also been invoked to describe susceptibility and resistance in many examples of experimental infection, and human disease. In the following sections, I will try to give an overview of some of the experiments which first suggested that cellular and humoral immunity were separable response mechanisms, and will briefly summarize the

relationships between antigen and lymphocytes which these experiments suggested. Investigations into variables of immunization influencing the immune response phenotype have led to contemporary models of CD4 T cell phenotype differentiation, which will be compared and contrasted in the conclusion of this introduction.

1.8.2 Kinetics of development of primary immune responses

In 1958, while studying the mechanism of DTH development in guinea pigs, Salvin noted several variables that affected the course of the immune response generated against diphtheria toxoid or OVA. Initially, Salvin observed that after injection of antigen in an oil and water emulsion, animals went through a latent period, followed by a period in which antigen-specific DTH reactivity was present in the absence of detectable antibody, and finally, after a further time interval, a shift was seen towards antigen-specific Arthus-reactivity (involving antibody-antigen complexes) and the development of circulating, antigen-specific antibody (Salvin, 1958). First, Salvin noted that increasing the dose of sensitizing antigen altered the kinetics of the periods described above; specifically, a decrease in the latent period as well as the period of DTH reactivity, and the "hastening of antibody formation". The converse observations were made when the sensitizing dose was lowered: increased latent and DTH periods and a significant delay in antibody formation. Finally, if antigen was injected in complete, mycobacterial adjuvant, specific DTH developed earlier and circulating antibody formation was "markedly delayed" (Salvin, 1958).

Salvin's research demonstrated that the kinetics of immune responses differed for different antigens (significant differences in the development of DTH and antibody responses against diphtheria toxoid and OVA), as well as between different doses of the same antigen, as well as between individual animals. Under the hypothesis that a given antigen is "homogeneous to the extent that it does not consist of two components, one of which causes delayed sensitivity and the other the Arthus type", Salvin concluded, "delayed sensitivity is a step in the development of the arthus [reaction]", and presumably circulating antibody (Salvin, 1958). This conclusion was similar to earlier findings of researchers investigating, in a far less controlled manner, immune responses generated against a variety of bacteria; Dienes for example suggested, "bacterial allergy [DTH] is a strong development of the early phase of the specific response to the antigen preceeding the production of circulating antibodies" (Dienes, 1935). A similar conclusion was reached by Benaceraff and Gell, attempting to verify Salvin's data usin a hapten-carrier system, who suggested that DTH reactivity was "an early, immature stage of immunity" preceding strong antibody reactivity (these researchers could not, however, demonstrate hapten-specific DTH during any phase of the immune response!) (Benaceraff and Gell, 1959). Salvin's early, comprehensive study of the kinetics of an antigen-specific immune response (generated with different antigens and different adjuvants), demonstrated the ability of the response phenotype to change with time, or mature, and furthermore, made clear relationships between the 'shape' of the immune response and both the antigen dose and the type of antigen and adjuvant employed. These relationships would be expanded upon significantly by several researchers, as

will be discussed. Importantly, the finding that the predominant phenotype of an immune response could differ dramatically depending on the day of assay sometimes seems forgotten in today's research, as pointed out by Kelso, who warns of the potentially misleading information gathered by "snapshot" analysis of cytokines or effector mechanisms at one particular timepoint (Kelso, 1995).

Turk and Humphrey investigated further the hypothesis that DTH and antibody formation represented two different stages "in a single process", as compared to the possibility that these two responses against antigen were "fundamentally distinct" (Turk and Humphrey, 1962). Guinea pigs rendered unresponsive (as measured by antibody production) to protein antigens, by administration of antigen during the prenatal and/or neonatal period, were found unable to mount DTH responses when challenged with the same antigen, in a manner known to induce antigen-specific DTH in control, untreated animals (for example, by administering a small antigen dose in CFA into the footpad). This result, using defined protein antigens, supported DTH and antibody responses as two points on the same 'response-continuum', demonstrating that, "the mechanism by which a state of delayed hypersensitivity, or antibody production, are elicited were not separable" (Turk and Humphrey, 1962).

1.8.3 Separation of cell-mediated and humoral responses

Arnold Rich, while studying tuberculosis patients in the 1930's and 1940's, noticed a trend of disparity between the degree of tuberculin hypersensitivity and the

titer of specific circulating antibodies in infected individuals/animals (see comment in Silverstein, 2002 and an extensive review of these observations in Rich, 1951 –originally published in 1941). This collection of observations might well be the most thorough documentation of a separation between cell-mediated and humoral responsiveness in animal models and human disease. Comprehensive studies on the relationship between antigen-specific cell-mediated and humoral response phenotypes would not be undertaken until roughly a decade after Rich's report.

In contrast to the views of researchers such as Salvin, and Turk and Humprey, discussed above, - Boyden, studying the possible "interference between the production of circulating antibody and the development of delayed-skin reactivity" obtained results demonstrating situations in which either of these two responses, but not both, could be generated. Guinea pigs injected with "unheated tuberculoprotein" three times in the course of 38-40 days before injection of a small challenge of BCG were unable to produce DTH reactivity when skin-tested, but instead displayed Arthus reactivity, while animals not pretreated before BCG infection mounted strong DTH (Boyden, 1957). Boyden discusses the possibility that circulating antibody and DTH are "produced by the same mechanism", the choice in effector phenotype determined by "the particular form of the antigen" (Boyden, 1957). As mentioned, this suggestion was in marked juxtaposition to the prediction that DTH represented a stage in antibody production, and represents the first experimental example of what would come to be known as 'immune deviation', as well as by a handful of other descriptive terms.

In the years following Boyden's experiments, certain clinical conditions were observed whose symptoms, including notable deficiency in antibody production (for example, agammaglobuglemia) or deficiency in DTH reactions (for example, Hodgkin's disease), led to the suggestion that, in contrast to Salvin's hypothesis, antibody formation and DTH reactivity represented separate, not necessarily associated, functional processes. Experimentally, humoral and cell-mediated immune responses were also shown to be somewhat disassociable. In the mid-1960's several groups began to find evidence for 'immune deviation', again borrowing Asherson and Stone's term, which seems to have stuck.

Asherson and Stone demonstrated this phenomena by first administering to guinea pigs a "deviating" injection of antigen given in alum, followed by the same antigen, given in CFA, a "fortnight" afterwards. After a further two weeks, animals were skin-tested for DTH with the same antigen in saline (Asherson and Stone, 1965). Consistently, the DTH reaction in 'deviated' animals was less, or absent, when compared to control, "un-deviated" animals. On the other hand, most experiments probing humoral responses of pretreated animals found only, "variable and slight changes in the titer of circulating antibody", (though a correlation between DTH and □2 antibody is made) – thus the term "immune deviation" (Asherson and Stone, 1965). This finding was repeated, and shown to be antigen-specific, using several different antigens. Using BGG, the authors also found that pretreatment with a relatively small amount of antigen (0.1mg compared to 1 or 10mg) had a lesser effect on the diminishment of DTH, supporting Salvin's observations on the relationship between antigen dose and strength of DTH. The authors conclude with

a brief discussion on the potential importance of immune deviation in certain autoimmune diseases, as well as in the development of lepromatus leprosy, potentially due to, "the conditions under which the immunological system first meets the immunogen" – very similar to Boyden's analysis (Asherson and Stone, 1965). Several other investigators published evidence showing similar examples of differential induction/regulation/tolerization of cellular and humoral immune responses during this period. Battisto and Chase, working with guinea pigs rendered tolerant to antigens ("hapten feeding"), found that antibody responses, but not DTH responses could be rescued after challenging animals with hapten-carrier conjugates in mycobacterial adjuvants. These results suggested, "that one immune response does not appear to be invariably linked to the other, instead, the available information suggests that they are separate responses, even though, at times, both may be directed against the same antigenic stimulus" (Battisto and Chase, 1965). A similar experimental system employing guinea pigs showed that antigen-specific (DNP-BGG) antibody, but not antigen-specific DTH reactivity, was observed when animals that had been exposed to the antigen neonatally were challenged with the same antigen in CFA at four weeks of age. Control, non-pretreated animals generated both DTH and antibody responses. The authors suggest that this "split-tolerance" represents "different immune processes which are not necessarily linked" (Borel et al., 1966).

In a series of studies detailing variables of induced non-responsiveness in adult guinea pigs, after systemic administration of a relatively large dose of antigen in saline, it was found that certain doses of "suppressing" antigen led to dramatic

decreases in antigen-specific DTH reactivity, while antibody responses remained unaffected, when animals were later challenged with antigen in complete adjuvant, compared to controls. Furthermore, some doses of suppressing antigen were found to enhance antigen-specific antibody production (□1), while all but eliminating DTH (Dvorak and Flax, 1966). Similar patterns were seen using rats (Dvorak et al., 1966). These experiments were perhaps more palatable in terms of studying 'real-life' DTH and antibody as they employed adult animals (in contrast to neonatal animals used by Borel et al.) and systemic "suppressing" procedures (in contrast to the oral route employed by Battisto and Chase). Evidence of the immune deviation phenomenon, the finding of an "inverse connection between the induction of immediate and delayed hypersensitivity" in mice was demonstrated in two separate experimental systems by Crowle and Hu, employing OVA as antigen (Crowle and Hu, 1965), and with both OVA and BSA as antigen (Crowle and Hu, 1966). These authors presented perhaps the most insightful conclusions of all the studies briefly described above, which will be discussed later.

Similarly, Loewi et al., found that pretreatment with antigen in incomplete adjuvant, or with no adjuvant, rendered adult guinea pigs unable to mount antigen-specific DTH reactions when the antigen was administered in CFA. This inhibition of DTH generally lasted over one month. Mechanistically, the inhibition of DTH responsiveness required a period of time to become established, as pretreatment had to be done at least two days before challenge in CFA (Asherson and Stone reported success with pretreatment one day before challenge). Furthermore, the authors suggested that, based on their findings that DTH and □2 antibody seemed to be

related (as Asherson and Stone reported), certain aspects of antibody formation and cellular immune functions might be related in terms of their induction (Loewi et al., 1966) - an interpretation quite different from Borel et al.'s idea of completely separate effector mechanisms, discussed above.

Evidence for immune deviation the-other-way-round – that is, DTH reactivity in the absence of detectable circulating antibody was also found. Guinea pigs pretreated with heat-killed mycobacteria could be rendered unable to produce antigen-specific antibodies in response to myobacterial antigens, when challenged four to five weeks later, while DTH reactivity remained unaffected, as compared to controls (Janicki et al., 1970). This study concluded that, "the animal's humoral response to ... antigens is suppressed, while its hypersensitive responses to the same antigens are unaltered".

In a follow-up study to his demonstration of immune deviation, Asherson probed the mechanism of immune deviation, finding that 'deviated' animals, in contrast to control animals, could not, after challenge with antigen in CFA, transfer antigen-specific DTH to naive animals. Furthermore, Asherson found no role for circulating antibody in down-regulating DTH in these transfer experiments (Asherson, 1966). These observations suggested a direct, functionally negative effect on the cells responsible for (the initiation of and/or mediation of) DTH by the 'deviating' antigen pretreatment. Borel's group further showed that lymphocytes, and not macrophages, obtained from deviated animals were responsible for the antigen-specific phenomenon, via several different *in vitro* experiments (Borel and David, 1970). More *in vitro* analysis of immune deviated lymph node cells showed

a decreased tendency for antigen-specific proliferation ("transformation"), compared to control animals, again showing a correlation between cellular response characteristics and DTH (Loewi et al., 1968). However, these experiments also showed that lymph node cells from animals that displayed no DTH reactivity could also proliferate in response to antigen. These studies highlight the difficulty in dissecting the mechanisms underlying the initiation of immune responses during the period preceding the development of the Two Signal Model.

1.8.4 The nature of the antigen influences the immune response phenotype

A series of experiments demonstrated that very small antigens, while capable of generating specific DTH reactions when given to adult guinea pigs in CFA, very rarely generated antigen-specific antibody (and when antibody was produced, at very low titers) (Jones and Leskowitz, 1965). This, and similar findings suggested that, "antibody formation and delayed sensitivity are separate processes, each favored by a different type of determinant" (Leskowitz et al., 1966). This analysis placed the uni-potential lymphocyte's decision to respond with either antibody or DTH, or both, only on the nature of the antigen, or in other words, was purely *qualitative* with regards to the immune system's effector mechanism choice. This model alone had great difficulty in describing the observations of immune deviation, seen with a variety of larger, more complex antigens.

In the period following the publication of the Two Signal Model (and the T cell – B cell collaboration experiments, discussed earlier, that cemented its foundations), more subtle investigation of variables influencing immune deviation, and more complete explanations of the relevance of these findings emerged. Pearson and Raffel investigated aspects of certain antigens, that when given to guinea pigs, could induce DTH responses, but could only stimulate antibody production very poorly, if at all (Pearson and Raffel, 1971). These experiments showed that, using a preparation of SRBC antigen obtained by incubating SRBC with (activated) macrophages and then harvesting the cell-free, released, degraded antigenic products as antigen ("digested macrophage contents"), "immunogenicity in respect to antibody induction declined with longer periods of intramacrophagic residence of SRBC [and] the ability to engender delayed reactivity appeared to increase". The authors conclude that, in addition to 'smaller' antigens, mentioned above, individual fragments of larger foreign antigens, and slightly altered self-antigens, containing few foreign determinants, might also preferentially induce DTH reactions. The proposed mechanism of regulating DTH/antibody phenotype, steeped in T cell – B cell collaboration, is centered on the ability of antigenic determinants to facilitate, "multi-cellular involvements" – an antigen containing only one determinant, or only T cell-specific determinants, while able to activate a T cell able to mediate DTH, would not facilitate the necessary T cell – B cell interactions needed to produce antigen-specific antibody (Pearson and Raffel, 1971). This model is still predominantly qualitative – the ability of the antigen to facilitate cooperation between different cells, thereby generating either DTH or antibody, dependent on the

antigen's determinants, but it leaves open the possibility of a more quantitative model, if the determinants facilitating cooperation between different cell types are identical: more determinants would favor more "multi-cellular involvements" and favor antibody production, while a limiting number of determinants would not allow these "involvements", over a certain threshold, resulting in DTH reactivity.

1.8.5 Immune deviation refined

A seminal body of work investigating immune deviation in more detail was amassed by Parish and colleagues during the late 1960's and the 1970's. Parish's experimental systems investigated both the influences of the relative antigenicity as well as the dose of a challenge in determining the phenotype of the immune response generated. Using increasingly acetoacetylated forms of the flagellin protein obtained from *Salmonella adelaide*, a procedure which modified the side-chains of specific amino acids, Parish showed a steady decline in this antigen's ability to bind flagellin-specific antibody, as well as a decline in the acetoacetylated forms to induce antibody specific for either native flagellin or acetoacetylated forms when given to adult rats (Parish, 1971a). When Parish assayed for cell-mediated immunity (DTH) against increasingly acetoacetylated flagellin, it was found that, "loss in the antigenic activity of flagellin [as assayed by antiserum reactivity] was accompanied by a reduced capacity of the molecule to initiate antibody formation but an enhanced ability of the protein to induce flagellin-specific cell-mediated immunity and antibody tolerance", as Pearson and Raffel suggested, demonstrating an "inverse

relationship" between these two response phenotypes (Parish, 1971b). A similar pattern was seen with a non-bacterial, more complex antigen; chemically modified SRBC, while still agglutinated by specific SRBC antiserum, lost the ability to induce SRBC-specific antibody, but increased in ability to generate DTH, when given both with and without CFA to adult rats (Parish, 1972).

In further agreement with Pearson and Raffel, Parish found that, compared to a similar dose of unmodified flagellin, digested forms of the antigen (via two separate procedures) – resulting in various smaller peptides, lost their ability to stimulate antibody formation, and instead induced strong DTH, (Parish, 1971b) while polymerized flagellin stimulated only antibody production (Ichiki and Parish, 1972). Furthermore, too great a loss in flagellin's antigenicity, via modification, resulted in a significant impairment of both humoral and cellular responses (Parish, 1971b). The ability of peptide to induce DTH against a subsequent challenge of whole protein antigen was seen in several other experimental systems, while still other systems showed no ability of peptide to stimulate protein-specific DTH, suggesting that not every protein fragment was a capable inducer of cellular immunity (reviewed briefly in Ichiki and Parish, 1972). A model put forth to describe these observations rejects the possibility that "the antigenic determinants for humoral and cell-mediated immunity differ" and instead proposes that both immune responses are directed against the same determinant, but determined by the degree of "specificity" of antigen-receptor interactions: less specific interactions favoring cell-mediated responses and more specific interactions favoring humoral immunity (Parish, 1971b). Put another way, DTH reactivity and humoral antibody production

could be viewed as, "competing immunological processes" influenced by "affinity of antigen for receptors" (Ichiki and Parish, 1972). Incorporating modern understanding of leukocyte subsets and their functions, Parrish suggests that antibody tolerance exists at the "B-cell level", while classic immunological tolerance, produced, for example, by neonatal antigen challenge, in which both antibody and DTH responsiveness is absent, exists "at the level both of the 'T' cell … and B cells" (Parish, 1971b).

An examination of the effect of antigen dose on the cell-mediated – humoral response phenotype revealed further complexities. It is worth noting that Parish employed a system ideal for studying the effect of dose because flagellin is a potent antigen, - very small, accurately measured amounts of the various flagellin preparations could produce measurable immune responses without a need for further adjuvant, and the antigen was rapidly eliminated from circulation. Parish investigated the state of DTH reactivity in rats immunized with relatively high and low doses of antigen, classically found to produce "high zone" and "low zone" tolerance (Mitchison, 1964). In rats displaying high and low zone antibody tolerance, after a 28 day immunization regime with varying doses of digested flagellin, significant DTH reactivity was observed while in rats injected with 'medium' doses of antigen, displaying antibody, no DTH was observed (Parish and Liew, 1972). This "mirror-image" relationship was far more pronounced in one strain of rat, demonstrating a genetic component in shaping the immune phenotype in response to a specific-antigen. The observations in this study are synthesized into Parish's existing model: "low doses of … flagellin would preferentially induce

delayed-type hypersensitivity … as the probability of small quantities of antigen inducing cell-to-cell interaction is low … higher doses of antigen would be expected to favor cell-to-cell interaction and therefore antibody formation" (Parish and Liew, 1972).

Parish's work resulted in the first comprehensive model describing the immune system's ability to choose between a predominantly cell-mediated or humoral response. This model, recognizing T cell –B cell collaboration as necessary in the production of antibody, states that availability of antigenic determinants allowing T cell – B cell interaction will result in antibody formation, while limiting antigenic determinants not allowing cellular interactions will result in only T cell activation and DTH responses (no cooperative mechanism is envisioned in T cell activation). An antigen with few recognizable determinants, or a low dose of antigen, will thus be unable to generate antibody production, but will efficiently induce DTH. This hypothesis would be refined significantly with the discovery of T cell subsets. Bretscher, incorporated a similar mechanism of immune class regulation into his general *self-nonself discrimination theory*, replacing Parish's "cell-to-cell interactions" with the level of "signal two", and thus the amount of associative antibody (T-cells) able to interact with antigen, as the prime variable affecting the response phenotype (Bretscher, 1974). Bretscher also speculates on the "biological significance of the hierarchy of immune responses", suggesting a meaningful relationship between the conditions required for the induction of, and the nature of the effector functions of both cell-mediated and humoral immunity: an antigen or pathogen with a low number of foreign sites, against which humoral (IgG)

immunity would generally not be effective, should induce cell-mediated immunity, which would be more effective against a threat with a low number of foreign sites, by virtue of the relatively few antigen-specific T cells present. On the other hand, comparatively more specific-T cells would be present upon challenge with an antigen containing many foreign sites, against which humoral antibody is generally more effective against. Thus, the inverse relationship seen between cell-mediated and humoral immunity seems to reflect teleological considerations (Bretscher, 1974).

1.9 The immune deviating cell

1.9.1 T cells control the cell-mediated/humoral decision

In 1974, Parish and Liew published hapten-carrier experiments showing that when rats were pre-immunized with different forms of flagellin, the ability of animals to produce hapten-specific antibody in response to a hapten-flagellin challenge 28 days later was determined by the anti-carrier response generated by the first challenge (Liew and Parish, 1974). Specifically, it was demonstrated that immunization with monomeric flagellin or acetoacetylated forms, the amount of hapten-specific antibody was dramatically decreased upon DNP-flagellin challenge. This result suggested that either different populations of T cells provided help for antibody synthesis and the development of DTH, or that the same T cell, when present in low numbers, favored DTH development, and when present in higher numbers, favored antibody formation (Liew and Parish, 1974). Similar studies led to

the finding that pre-treatment of guinea pigs with a dose of hapten-carrier, which resulted in both carrier- and hapten-specific antibody, rendered animals unable to mount carrier-specific DTH when challenged with a normally DTH-inducing challenge of conjugate in complete adjuvant (Nevea and Borduas, 1974). Like Parish, these authors appreciated that "T cells are directly involved in delayed hypersensitivity and [that] interactions between T cells and carrier are necessary for antibody synthesis", suggesting that immune deviation is centered on antigen-specific T cells. These early studies, combining hapten-carrier systems in immune-deviating conditions showed that it was possible to "dissociate the two known functions of T cells: cellular immunity and helper function [in antibody formation]" (Nevea and Borduas, 1974).

Defining the role of T cells in immune deviation further, it was demonstrated that when whole spleen cells or Ig(-) spleen cells were transferred from a mouse immunized to respond against horse RBC (HRBC) with a strong humoral phenotype, to a naive mouse, just before immunization with HRBC in a manner which leads to the generation of strong HRBC-specific DTH, the antigen-specific DTH is suppressed (Ramshaw et al., 1976). This effect was not seen with the transfer of spleen cells from naive mice, nor with the transfer of Ig(+) spleen fraction of humorally immune mice, nor could it be transferred with HRBC-specific antibody. Further studies defined conditions where T cells that suppressed HRBC-specific antibody responses were induced (Ramshaw et al., 1977a). These conditions were found to favor the generation of antigen-specific DTH responses. Finally, it was demonstrated that T cells responsible for the transfer of DTH suppression, and T

cells transferring suppression of antibody responsiveness could be separated in terms of Ly antigen expression: suppressors of DTH were found to be Ly1+ (CD4) while suppressors of antibody responsiveness were characterized as Ly2+ (CD8) cells (Ramshaw et al., 1977b). These studies extended the role of T cells from involvement in the initial generation of immune deviated responses, also to the maintenance of immune deviated states.

1.9.2 Helper T cells defined

The developments described earlier under section 1.6.4 solidified Ly1+ T cells as helper cells involved in both antibody and DTH responses (though Ly2+ cells were also found involved in certain cell-mediated systems). However, it still was unclear whether antigen-specific helper cells involved in DTH and antibody responses originated from a common precursor or whether they represented completely different Ly1+ T cell populations. Experimental evidence for the heterogeneity of helper T cells accumulated in the late 1970's with observations from several groups of functionally different T helper cells in antibody formation, which could be separated on the basis of nylon wool adherence and Ia expression (briefly reviewed in Bottomly, 1988). These studies employed polyclonal T cell populations. What followed this period of initial T helper cell research has been called by Liew "the 'dark ages' of cellular immunology" – in large part because technology could not yet allow the detailed analysis of individual cells, nor their isolated products, (Liew, 2002).

The introduction of single-cell cloning of T cells, and the advent of sensitive cytokine assays in the early 1980's allowed a new approach to study CD4 T cells and their helper abilities. Using long-term T helper clones specific for SRBC and HRBC, it was shown that these cells, isolated for their ability to help antibody production, could also mediate DTH against the same antigen, formally demonstrating that "helper T cells can mediate DTH responses" (Bianchi et al., 1981). This seemed to support the theory that DTH and antibody responses were both helped by the same cell, in contrast to Parish's findings and the suggestion that different cells (either derived from different precursors, or via differential, polarizing activation signals received by a common precursor), but at different anatomical sites, as summarized here by Lagrange and Mackaness's suggestion that "diversion of reactive cells from circulation to undertake an alternative role in antibody formation in the spleen" might account for immune deviation (Lagrange and Mackaness, 1978).

It was later shown, with human allo-reactive T cells, that the same T cell clone could help both B cell antibody production and CTL responses (Rimarli et al., 1984), although a similar study found two "functionally restricted" helper T cells – one providing help for B cell responses, and the other involved in the generation of allo-specific CTL responses (Friedman and Thompson, 1983). Further experiments, using monoclonal antibodies to separate the T cell pool into subsets, found that one subset (OX-22$^-$) produced IL-2 but displayed poor helper activity (B cell help), while the OX-22$^+$ CD4 T cells produced little IL-2 but helped B cells produce antibody (Arthur and Mason, 1986). This finding led to the interpretation that separate CD4 T cell populations could help either antibody production (OX-22$^+$) or the IL-2-

dependent generation of CTLs. In fact, one year before the first publications on Th1/Th2 CD4 T cell subsets, Arthur and Mason, summarizing a discussion of the roles of OX-22$^{+/-}$ T cells suggest that "the existence of different T cell subsets providing help for humoral and cell-mediated responses provides the possibility of regulating these two arms of the response independently" (Arthur and Mason, 1986 – received for publication in Oct. 1985). These and other conflicting observations on the nature of T helper cell function/regulation left the field of immunology with a gaping void in terms of a focus for investigating how the variables of immunization described by Parish and by earlier studies of immune deviation, were translated into regulated, phenotypic response choices by cells of the adaptive immune system. The discovery of Th1 and Th2 T helper cell clones would revolutionize this understanding and lead to powerful strategies of regulating the phenotype of immune responses by vaccination and immunotherapy.

1.10 Th1/Th2

1.10.1 Polarized CD4 T cell clones

In 1986, panels of long-term, antigen-specific, MHC II restricted murine CD4 T cell clones were described with stable ("no evidence of interconversion ... between") and distinct phenotypes based on antigen-specific (or ConA stimulated) cytokine secretion patterns (Mosmann et al., 1986). Interestingly, the authors chose to keep the terminology proposed by an earlier study of helper cell heterogeneity (Tada et al., 1978). Th1 cells were described as producing IL-2, IFN-γ, GM-CSF, and IL-3, while Th2 cells were characterized by production of IL-3, IL-4 (BSF-1) and IL-5 (Mosmann et al., 1986). Functional differences were also seen between the two types of helper cells: Th1 cells, but not Th2 cells were found to mediate specific DTH responses upon transfer into naive mice in combination with a variety of specific antigens (Cher and Mosmann, 1987) – supporting the earlier observations of Bianchi's group. Th1 and Th2 cells were also shown to affect humoral responses differently; some Th1 clones were able to help B cells produce antibody, predominantly IgG2a, while Th2 clones led to the production of predominant IgG1 *in vitro* (Stevens et al., 1988). The ability of Th1 clones to help in antibody formation was dependent on the number of Th1 cells in culture and the amount of IFN-γ produced – too much inhibited antibody production completely (Street and Mosmann, 1991). This finding could account for the observations that some, but not all Th1 clones could help antibody synthesis, and why, *in vivo*, strong Th1 responses

sometimes had no antibody component. Antibody formation could be decreased dramatically, but not totally, in this system with the addition of antibody specific for either IFN-γ (Th1) or IL-4 (Th2), suggesting that T helpers influenced the regulation of antibody production both at the cytokine level and via another mechanism, perhaps involving cell-to-cell contact.

Thus, by the close of the 1980's, two separate antigen-specific CD4 T cell populations seemed capable of generating either a predominant cell-mediated response, characterized by DTH and little antibody production (Th1), or humoral response featuring high levels of antibody as well as mast cell and eosinophil activities (Th2) when challenged with the same antigen. Though most antigen-specific responses reflected a definite mixed Th1/Th2 response – both in terms of overall immune phenotype and CD4 T cell clones obtained, the *in vivo* relevance of polarized responses was highlighted in a murine leishmaniasis model. In this model, in response to a challenge with an arbitrary but high number of parasites, susceptible mice (BALB/c) succumbed to a progressive disease, displaying high levels of antigen-specific antibody and IL-4 production and low IFN-γ and DTH reactivity, while resistant mice (C57Bl/6) contained the parasite with a response characterized by low antibody and IL-4 production but high amounts of IFN-γ and DTH reactivity (Sadick et al., 1986; Locksley et al., 1987). Furthermore, it was demonstrated that antigen-specific Th1 clones could protect naive BALB/c mice from a normally fatal parasite challenge, while Th2 clones worsened disease (Scott et al., 1988). This study represents the first correlation of disease outcome with CD4 T cells of defined Th1/Th2 phenotype. This topic will be explored in more depth later. Other early *in*

vivo studies correlated the outcome of 'high dose' tolerance, following similar protocols as early investigators of immune deviation, with the selective unresponsiveness of Th1-like (cytokine and antibody isotype) effector mechanisms but unchanged or enhanced antigen-specific Th2-like responses (Burstein et al., 1992; DeWit et al., 1992; Vidard et al., 1995).

The experimental clarity of the nature of protective and non-protective CD4 T cells was clouded by the demonstration of antigen-specific "multiproducer" CD4 T cell clones, termed Th0 clones, derived from shorter-term cultures, which represented roughly 30 percent of antigen-specific T cells (Firestein et al., 1989, Street et al., 1990). These Th0 cells produced both Th1 and Th2 cytokines in response to a variety of specific antigens, but tended after longer periods in culture, to slide towards the conventional Th1 or Th2 phenotype, perhaps explaining the relative absence of Th0 cells in earlier studies employing long-term clones. The Th1/Th2 concept was further found wanting with the observation that naive polyclonal T cells could be stimulated to mature from a stage in which only IL-2 was secreted at high levels, to a stage in which IFN-□ and IL-4, as well as other cytokines, but not high levels of IL-2 were produced (Swain et al., 1988). Swain's group termed the IL-2-producing cells 'precursor' T cells, as compared to 'effector' T cells, which developed four to five days after culture.

Combined, these observations suggested that CD4 T cells progressed from an IL-2-producing precursor stage, through a non-committed Th0 stage upon antigen activation, and finally, after a longer period of stimulation, into either polarized Th1 or Th2 phenotypes. Using an allo-antigen system, evidence that either a polarized

Th1 or Th2 cell could develop from IL-2 producing clone, under the influence of appropriate cytokines, supported this mechanism, although little evidence for a Th0 stage was seen (Mosmann and Sad, 1994, Wang and Mosmann, 2001). This could be because of the extreme Th1/Th2 polarizing conditions in culture. Important issues raised by this and other studies, which were not addressed by early Th1/Th2 investigators, included what type of variables favored the generation of Th1 versus Th2 cells upon primary antigen encounter *in vivo*. Many early studies concentrated on the APC involved in antigen presentation to a precursor T helper cell, with conflicting views (reviewed in Mosmann and Coffman, 1989). Also, the question of whether Th1 and Th2 cells originated from a common precursor, or from different Th1 and Th2 predisposed precursors was only speculated upon. Furthermore, the demonstration of clear cut Th1 and Th2 clones amongst those generated from human CD4 T cells was not readily apparent in early studies, calling into question the correlation between Th1/Th2 phenotype and disease outcome. With the resolution of this debate, by the clear demonstrations of Th1/Th2-like helper phenotypes in human cells (reviewed in Romagnani, 1991), the Th1/Th2 paradigm achieved further legitimacy. Thus, the prediction of Waksman, made in 1959: "is it not possible that treatment which leads the immunologically competent cell to make humoral antibody makes this cell unable to develop delayed hypersensitivity" (Waksmann, 1960) was shown to be largely correct, when applied to CD4 T cell dependent immune responses.

1.10.2 Th1/Th2 paradigm lost?

Careful investigation at the single-cell level often leads to conclusions that blur the classic 'textbook' Th1/Th2 cytokine-based phenotype of a response seen at the population level. This complexity of the Th1/Th2 paradigm was revealed when the relatively clear-cut Th1, Th2 and even Th0 cytokine-secretion profiles of established antigen-specific clones were found unable to broadly characterize the antigen-specific CD4 T cells generated after primary immunization *in vivo* (reviewed by Kelso, 1995). Kelso argues that "the current terminology that appears to equate 'Th1' and 'Th2' responses with Th1 and Th2 cells is therefore misleading", because, often, very few cells display the canonical combination of Th1 or Th2 cytokines, and can sometimes even co-express cytokines from the two profiles (Kelso, 1995). For example, using a sensitive RT-PCR technique, it was demonstrated that although on a population level, an allogenic response *in vitro* could be classified as 'purely' Th1, based on high IFN-γ production and no detectable IL-4 by ELISA, on the single-cell level, ten percent of cells expressed one or more Th2-type cytokines (Kelso et al., 1999). Similar patterns were observed in a predominant Th2 response. It was also shown, using an *in vitro* system employing transgenic CD4 T cells, that in a response described as Th0 on the population level, a minority of individual cells co-expressed mRNA for a given pair of cytokines typical of a Th0 phenotype (Bucy et al., 1995).

Thus, it seems that often the terminology of Th1/Th2 is valid to describe the nature of the response at the population level, or, in other words, that the "Th1 or Th2 profile appears to represent the average phenotype of the population" of

responding cells (London et al., 1998), while at the single-cell level, the story is more complex and less clear-cut, and that this complexity is increasingly revealed with increasingly sensitive assays. While this argument is valid in many situations, the Th1/Th2 paradigm is still a powerful way to assess disease and correlates of protection. After all, it is the response of a population of antigen-specific cells, and not the single responses of a collection of individual cells, which will protect or not protect against a given challenge. However, while this argument against the theoretical validity of the Th1/Th2 paradigm is "in one sense ... a question of semantics" (Kelso, 1995), it is critically important when dealing with models of initial CD4 T cell phenotype acquisition. For example, Kelso's observations suggest that the theoretical precursor CD4 T cell -to IL-2 producing CD4 T cell -to Th0 CD4 T cell -to either Th1 or Th2 CD4 T cell roadmap is invalid at the single cell level, and is not controlled solely by polarizing, binary signals such as IL-12 and IL-4. Some *in vitro* data suggests that the generation of a stable Th0-type phenotype (as assessed after 9 days of culture by intracellular cytokine staining) might require specific cytokine mileus at priming, and multiple, successive encounters with antigen (Miner and Croft, 1998); this finding suggests that the generation of long-term, non-classical Th1/Th2 cytokine profiles amongst CD4 T cells might be the result of certain culture conditions, or relatively rare situations of antigen encounter *in vivo*. Nevertheless, the multitude of cytokine-producing patterns seen amongst responding CD4 T cells in some examples of responses that are Th1/Th2 polarized on a population level, suggests a stochastic element in Th1/Th2 lineage commitment.

This concept has been elaborated upon recently, offering an alternative model of Th1/Th2 phenotype maturation to the linear model described above. Briefly, this model suggests that a newly activated CD4 T cell has the capacity to respond with any cytokine profile, with no bias towards the Th1 or Th2 pole. From this initial pool of activated cells, a population of predominant Th1-producing or predominant Th2-producing cells will then be favored to outgrow based on the cytokine environment present, with IL-12 and IL-4 serving not so much as polarizing instructive cytokines, but as selective growth factors (Reiner and Seder, 1999; Coffman and Reiner, 1999).

In summary, while the Th1/Th2 paradigm is a powerful tool in the understanding of protective and non-protective immune responses, on a single-cell level, the definitions first obtained with long-term cultures of antigen-specific clones do not often apply. Thus, even in a single-cell assay, the detection of one prototypical Th1 cytokine versus one prototypical Th2 cytokine might not represent the actual number of polarized CD4 T cells, but might better offer an indication of the relative measure of the Th1 and Th2 components of a more complex response. Several variables have been found to influence the Th1/Th2 character of a primary immune response; important aspects of these variables will be discussed briefly, after introducing observations which actually extend the Th1/Th2 paradigm.

1.10.3 Paradigm expanded

There is considerable evidence that effector cells other than CD4 T cells can also adopt differential cytokine secretion patterns very similar to the standard Th1 and Th2 arrays. CD8 T cells have been classified as Tc1 and Tc2, in both humans and mice, based on predominant IL-2 and IFN-□(Tc1) or predominant IL-4 and IL-5 (Tc2) expression (reviewed in Mosmann and Sad, 1996). Furthermore, Tc1 and Tc2 phenotypes are associated with the same stability as Th1 and Th2 effectors. Tc2 CD8 T cells were originally produced *in vitro* by anti-CD3 stimulation and exposure to IL-4, in much the same manner as was used to generate Th2 clones (Seder at al., 1992). The presence of IL-12 in culture, but not of IFN-□, was found to significantly upregulate IFN-□production from antigen-stimulated transgenic CD8 T cells (Croft et al., 1994). Thus, typical Th1/Th2 polarizing cytokines (discussed below) seem to also drive the lineage commitment of CD8 T cells. Interestingly, in most experimental systems, Tc1 and Tc2 cells displayed comparable cytotoxic ability (Croft et al., 1994, Sad et al., 1995, Mosmann and Sad, 1996). The physiological role of separate Tc1 and Tc2 populations is not well understood, although an intriguing possibility is suggested by the observation in some instances of differences in tissue-specific homing between these two populations (Woodland and Dutton, 2003).

Knowledge of the immunobiology of □□ T cells has become increasingly complex. Interestingly, □□ T cells can also be polarized towards a Th1 or Th2 pole. Much like □□ CD4 T cells, □□ T cells have been shown to respond with either IFN-□

or IL-4 when mice are challenged with Th1 or Th2 stimulating pathogens *in vivo* (Ferrick et al., 1995). *In vitro*, the cytokines IL-12 and IL-4 can prime populations of murine and human □□ T cells to predominantly produce IFN-□ or IL-4, respectively (Yin et al., 2000, Wesch et al., 2001). A small subset of $CD4^+$ □□ T cells, present in both the mouse and human has been associated with predominant Th2-type cytokine production (Wen et al., 1998). At present, it is unclear if influences other than pro-Th1 or pro-Th2 cytokines can influence the Th1/Th2-like phenotype of □□ T cells.

NK cells have also been reported to, under certain conditions, fall into Th1/Th2 like phenotypic categories (NK1/NK2) (Peritt et al., 1998). Although originally restricted to human NK cells, very recent experiments have demonstrated the generation of murine NK1 and NK2 cells *in vitro* (Katsumoto et al., 2004). As in Th1/Th2 and Tc1/Tc2 differentiation, *in vitro* NK1/NK2 differentiation seems to be heavily influenced by the cytokines IL-12 and IL-4 (Katsumoto et al., 2004). Of note, NK2 cells produce IL-5 but not IL-4, but all subsets display an equivalent cytotoxic activity, similar to the findings made with Tc1/Tc2 cells. NK1.1+ T cells have also been described as polarized towards a Th1 or Th2 like cytokine secretion profile. Via CD1-dependent activation, NK T cells have been shown to be important in resistance against bacterial, fungal, parasitic, and viral challenges, acting either as a pro-Th1 or pro-Th2 element (reviewed in Hansen and Schofield, 2004). Generally, NK T cells recognize lipids and glycolipids, extending the range of T cell surveillance; the exact ligands recognized by these cells remains largely unknown, though an endogenous ligand, perhaps disregulated in some disease states, which is

recognized by mouse and human NK T cells has recently been described (Zhou et al., 2004). Porcelli's group has implicated co-stimulatory signals from APC present in distinct microenvironments as being critical in the induction of NK T cells secreting either Th1 or Th2 associated cytokines (Yang et al., 2003). Because NK T cells recognize glycolipid antigens in contrast to conventional peptide antigens, these cells could represent an alternative source of early IFN-γ or IL-4 production, which may be important in establishing a polarizing environment for conventional CD4 T cells in some, but not all, infectious situations. This more restricted view of the importance of NK T cell-derived cytokines, especially IL-4, in establishing a pro-Th1 or pro-Th2 environment, is supported by observations of strong Th2 responses in CD1-deficient mice (Smiley et al., 1997). Similarly, no appreciable role for NK cells or NK T cells was found in influencing the induction of Th1 or Th2 OVA-specific responses (cytokines and antibody isotype) when these subsets were depleted in several strains of mice (Wang et al., 1998) or when β2-microglobulin-deficient mice were employed (Guery et al., 1996). A Th1/Th2-like polarization has also been documented in models studying the Vβ14+ NK T cell subset – though quite prolonged protocols of antigen exposure (alpha-galactosylceramide) were needed to observe development of a Th2-like phenotype (Taniguchi et al., 2003).

1.10.4 The importance of the phenotype of the immune response in the context of the Th1/Th2 paradigm

To jump backwards for a moment to the work of Parish, - in a review charting the history of his personal canon on immune deviation, Parish notes that both he and some of his reviewers were concerned as to the physiological relevance of the experimental phenomena (Parish, 1996). He further reports that he was solaced by several observations in clinical cases of leprosy, in which predominant cell-mediated or humoral responses could often be correlated with protection and worsening of disease (reviewed in Turk and Bryceson, 1971). Similarly, as discussed earlier, several earlier investigators closed their discussions with predictions that experimental immune deviation might relate to the clinical outcome of mycobacterial infections (Boyden, 1957, Asherson and Stone, 1965, Crowle and Hu, 1966). George Klein, in 1968, bluntly stated his belief that immune deviation was critical in protection from cancer, "It will be most important to establish what variables of ... immunization ... dosage, route of administration, and timing (of antigen administration) are critical to achieve the objective, which seems to be a stimulation of host cell-mediated rejection with a minimum risk of antibody-mediated enhancement" (Klein, 1968).

While correlating examples of 'split-tolerance' with susceptibility or resistance to certain infections represented a major step forward in understanding pathogen-host interactions, it was not fully appreciated by the immunological community until the discovery of Th1/Th2 CD4 T cells. The observation that the

protective effectiveness of an immune response often depends not only on its magnitude and specificity, but also on the predominance of either Th1- or Th2-centered effector mechanisms employed, brought with it new criteria for investigating the immune system's role in health and disease; there are now numerous examples, in several different animal models and in human studies, demonstrating the importance of the Th1/Th2 phenotype of the response in the outcome of many specific pathogen challenges, in protection against certain cancers, and in several autoimmune disorders (see Mosmann and Sad, 1996, Bretscher et al., 2001, Moss et al., 2004). Specifically, strong Th1 responses have been found to correlate with protection in human patients against several important intracellular pathogens, including those causing leprosy, tuberculosis, HIV/AIDS, the leishmaniases, and HCV, while Th2-associated responses often correlate with progressive or chronic disease (Turk and Bryceson, 1971; Lienhardt et al., 2002; Clerici and Shearer, 1993; Hailu et al., 2001; Tsai et al., 2003). Predominant Th2 responses have long been known to protect against bacterial toxins (see 1.2.1), circulating viruses (neutralizing antibodies), and extracellular pathogens, most notably parasitic worms (Finkelman and Urban, 2001).

The importance of inducing, or imprinting, an antigen-specific response with the correct, protective Th1/Th2 phenotype was predicted by Crowle and Hu in 1966 with remarkable clarity:

> Once an animal has been exposed to antigen ... it differs from the pristine state not only ... in being capable of subsequent magnified responses to the antigen (anamnesis) but also ... in the opposite fashion of being less than normally able to develop some kinds

> of [responses]. The ultimate response of any immunized animal should be viewed not only as induction of one or another kind of response, but also as the outcome of a balance struck between possibly competing types of immunologic responses (Crowle and Hu, 1966)

This insight, as does Klein's regarding cancer resistance, holds extreme relevance for vaccination. The design of an effective vaccine must not only imprint antigen-specificity, but also must imprint the correct response phenotype, dictated by the particular pathogen being vaccinated against. Several variables have been found to alter the Th1/Th2 phenotype of a primary immune response generated both *in vivo* and *in vitro* – these variables will be discussed below in some detail.

1.11 Exploring variables influencing Th1/Th2 phenotype

1.11.1 Variables affecting Th1/Th2 phenotype: cytokines

Early *in vitro* studies of the modern era (late 1980s and 1990s) addressed the roles of different cytokines in the development and maintenance of established Th1/Th2 clones, as well as the development of polyclonally stimulated cells *in vitro*. It was observed that IFN-γ could inhibit the proliferation of established Th2 clones whereas no negative effect was seen on the growth of Th1 clones (Gajewski et al., 1988). Furthermore, this group found that when IFN-γ was added to cultures of short-term antigen-specific clones, a high proportion (roughly two thirds) developed a Th1 phenotype compared to a very low proportion (roughly five percent) when IFN-γ was absent from cultures (Gajewski et al., 1989). This finding was extended

in vivo employing the murine leshmaniasis model (Scott, 1991; Coffman et al., 1991). Furthermore, it was observed that IL-4 producing Th2 cells were preferentially generated after culture of naive T cells stimulated with anti-CD3 only if IL-4 and IL-2 were present in cultures; this combination of cytokines led to the generation of roughly 100-fold higher IL-4 compared to control cultures, and the effect of IL-4 was not inhibited by the presence of IFN-γ (LeGros et al., 1990, Swain et al., 1990a and b). A similar effect of IL-4 favoring Th2 responses was also seen *in vivo*, again using the murine leshmaniasis model as well as in murine nematode infections, where the administration of IL-4 was found to cure established infections, in part through a T and B cell-independent mechanism (Chatelain et al., 1992; Urban et al., 1991; Else et al.; 1994, Urban et al., 1997). Similar patterns of Th1/Th2 development and reciprocal regulation of phenotype by IFN-γ and IL-4 were observed with human T cells (Maggi et al., 1992). These and other, similar results employing a variety of *in vitro* systems (reviewed in Mosmann and Coffman, 1989) suggested that IFN-γ and IL-4 might play important roles in the polarization of ongoing responses, but, as pointed out by LeGross et al., this mechanism might not apply to the initial activation of CD4 T cells as these polarizing, T cell-produced cytokines might not be available "in the locations or in the amounts required for the induction [of Th1/Th2 phenotype]" (LeGross et al., 1990).

A refined model for a cytokine-based determination of Th1/Th2 phenotype was suggested by the characterization of IL-12, and the finding that IL-12 could cause outgrowth of Th1 CD4 T cells when added to cultures of human peripheral blood mononuclear cells (PBMC) in a similar manner as IFN-γ, even when

neutralizing antibody to IFN-γ was added to culture (Parronchi et al., 1992, Manetti et al., 1993). A role for another 'innate' cytokine in influencing the Th1/Th2 phenotype of the immune response was observed *in vivo* when treatment of mice with IFN-γ followed by antigen challenge decreased the amount of IL-4 mRNA and increased the amount of INF-γ mRNA compared to controls (Finkelman et al., 1991). As both IL-12 and IFN-γ are cytokines produced by MHC II bearing APC (as well as others), especially macrophages, Romagnani suggested that, "given the capacity of viruses and intracellular bacteria to stimulate macrophage production of IFN-γ and IL-12, T_H cells may be simultaneously presented with processed antigen plus cytokines that induce them towards a Th1 phenotype" (Romagnani, 1992). Similarly, in this brief article, Romagnani suggests that mast cells and/or basophils might represent initial T cell-independent, polarizing sources of IL-4 for Th2 development.

Support for this concept, strongly influenced by Janeway's model of T cell activation, was obtained in a murine system as well. TCR transgenic CD4 T cells preferentially developed a Th1 phenotype when macrophages, activated by *Listeria monocytogenes*, were present in culture (Hseih et al., 1993a, Hsieh et al., 1993b). The Th1-biasing effect was shown to be solely due to IL-12 as IL-12 could replace activated macrophages in this system, as long as other MHC II+ APC were present, and the effect of activated macrophages in culture was not inhibited by MHC II mismatch with responding T cells. A direct effect on precursor T helper cells was also shown by stimulating naive CD4 T cells with anti-CD3 in the presence or absence of IL-12 *in vitro* (Seder et al., 1993). However, IL-12 also induces potent

IFN-γ production from NK cells (Kobayashi et al., 1989), suggesting that IL-12 might also act in an IFN-γ-dependent mechanism to bias initial Th1 development.

Support for this spectrum of effects produced by IL-12 was obtained with the characterization of the IL-12 receptor, which is expressed on NK cells, TCR-activated T cells, and some DC subsets (Presky et al., 1996, Trinchieri, 2003). In further support of a pro-Th1 cytokine environment, stimulation of activated T cells with IFN-γ, IFN-α, TNF, and IL-12 itself can induce upregulation of the IL-12 receptor and increased responsiveness to IL-12 (Trinchieri, 2003). *In vivo* evidence for a role of IL-12 in establishing Th1/Th2 phenotype was observed by treating mice with IL-12, which protected susceptible strains from an otherwise lethal challenge of *Leishmania* parasites, and treatment of resistant strains with anti-IL-12 was found to exacerbate disease (Sypek et al., 1993). Importantly, a role for IL-12 in shifting the response towards a predominant Th1 phenotype was observed in a non-infectious experimental system, employing TNP-KLH (McKnight et al., 1994).

Some evidence has been interpreted as suggesting that IL-12 exerts its effects in the generation of, and not necessarily during the effector phase of a Th1 response; this was demonstrated most convincingly *in vivo* using a murine *Toxoplasma gondii* model, where IL-12 production was required for the establishment of a protective Th1 response, but neutralization of IL-12, once a Th1 response was established, had no negative effect (Gazzinelli et al., 1994). IFN-γ was shown to be the protective Th1 response element in ongoing, established *Toxoplasma* responses (Scharton-Kersten et al., 1996). However, other studies have shown that IL-12 production is critical during the effector phase of many responses, including protective responses

against *L. major* and *T. gondii* (Park et al., 2000; Yap et al., 2000). These experiments were conducted employing IL-12 deficient hosts in which infection was contained effectively when exogenous IL-12 was given to animals, but protection waned quickly when IL-12 was removed.

A further innate cytokine, similar in structure to IL-12, termed IL-18, has also been shown to promote IFN-γ production from Th1 T cells and NK cells in the mouse and human, and has been shown to synergize with IL-12 in causing maximal IFN-γ production in several systems (O'Garra and Robinson, 2004). However, IL-18 cannot substitute for IL-12 in the initial development of the Th1 phenotype *in vivo*, and these two cytokines have been shown to signal through different intracellular pathways, indicating some non-overlapping signaling events (Robinson, 1997). The exact roles of IL-18, and other novel pro-Th1 cytokines including IL-27 and IL-23 – which seems to have a more pronounced effect on Th1 'memory cells', has not been established *in vivo* (O'Garra and Robinson, 2004; Trinchieri, 2003).

A pro-Th2 cytokine environment has been typified as the early presence of IL-4, and, in some cases, a second Th2-polarizing cytokine, IL-10. This second regulatory Th2-associated cytokine, in addition to IL-4, was characterized by Mosmann's group in 1989. IL-10 was shown to inhibit cytokine production by Th1 clones when added to cultures of clones stimulated with antigen and APC, but not when stimulated with non-specific ConA or anti-CD3 (Fiorentino et al., 1989). The effect of IL-10 on down-regulating Th1 responses is largely due to the inhibition of IL-12 production by professional APC (D'Andrea et al., 1993, Murphy et al., 1994). In support of this hypothesis, the addition of IL-12 to cultures of APC-stimulated

CD4 T cell clones can restore substantial Th1 cytokine production, even in the presence of IL-10 (Mosmann, 1994). IL-4 was the first cytokine shown to have pro-Th2 activities, as already discussed. In addition, IL-4 was found in both mouse and human to down-regulate expression of the IL-12 receptor on T cells (Rogge et al., 1997, Szabo et al., 1997). Further evidence for the biasing effect of IL-4 was observed in a system employing DC as APC for OVA-specific TCR transgenic mice (Macatonia et al., 1995). This study was the first to demonstrate that DC could produce physiologically relevant Th1/Th2 biasing cytokines, in this particular case, IL-12. However, the Th1-polarizing effect of purified DC was only seen upon the neutralization of IL-4 in culture – the very small amount of endogenous IL-4 produced by the sorted naive transgenic CD4 cells seemed able to bias the response towards the Th2 pole, which could be overcome with the addition of exogenous IL-12 (Macatonia et al., 1993). This observation supports other studies, employing a variety of systems, concluding that IL-4 produced by CD4 T cells (including the possibility of NK T cells in certain, but not all, situations), and not an innate source of IL-4 (activated basophils or mast cells, for example) is critical in Th2 biasing (Schmitz et al., 1994; Gollob and Coffman, 1994; Smiley et al., 1997; Delespesse et al., 1998). It is worth noting that in certain situations, IgE production, a hallmark of the Th2 response, can be generated in IL-4-deficient mice, suggesting the existence that an IL-4-independent mechanism of Th2 polarization, possibly involving cell-surface molecules (Morawetz et al., 1996). In fact, some evidence supports the position that early IL-4 production, "is not required to induce naive $CD4^+$ T cells to produce the initial IL-4 response, but rather amplifies later IL-4 production" (Morris

et al., 1998). Some data has implicated IL-9 production, a lesser-mentioned cytokine of the prototypical Th2 profile, as being an important constituent of a pro-Th2 environment *in vivo* (Arendse et al., 2005). As this study was done using a murine leishmania system, it is possible that the effects of IL-9 reported are pathogen-specific and not universal.

Thus, the Cytokine Environment Hypothesis proposes that a pro-Th1 cytokine environment, highlighted by early production of IL-12 from APC, and in some situations the consequent early production of IFN-γ by NK cells, could potentially cause direct pro-Th1 effects on precursor CD4 T cells and their proliferation via IL-12, and hinder the proliferation of potential pre-activated Th2 responders, via IFN-γ. A pro-Th2 environment, characterized by IL-4 and IL-10 production by innate and/or and early IL-4 production from activated T cells, would down-regulate both IL-12 produced by APC and IL-12 receptor expression on T cells, inhibit the extent of subsequent IFN-γ production, and favor the outgrowth of Th2 polarized clones. Several other cytokines also affect the Th1/Th2 polarizing environment to a lesser extent, including TGFβ, which also functions in down-regulating IL-12 production (Trinchieri, 2003). The Cytokine Environment Hypothesis is complicated further by the finding that different APC populations, and the different co-stimulatory molecules they may express upon activation, can also profoundly affect Th1/Th2 phenotype generation. These influences on the Th1/Th2 phenotype of CD4 T cells will be discussed in the following two sub-sections.

1.11.2 Variables affecting Th1/Th2 phenotype: the antigen-bearing APC

Early studies suggested that Th1 cells might preferentially be expanded by B cells, and Th2 cells by macrophages, due to cytokines produced by these APC. However, because of the more physiologically implicit relationships between Th1 cells and macrophages (DTH) and Th2 cells and B cells (humoral responses), the reverse prediction was also made (Mosmann and Coffman, 1989). *In vitro*, B cells were found to optimally cause the proliferation of Th2 clones, and macrophages of Th1 clones (Gajewski et al., 1991). The advent of DC as the prime APC involved in T cell activation naturally focused a great deal of research on their ability to generate either Th1 or Th2 effector CD4 T cells.

In mice, lymphoid derived CD 11c^{+}, CD8□□$^{+}$ and myeloid derived CD11c^{+}, CD8□□$^{-}$ DC, when loaded with relevant peptide antigen, were found to cause similar proliferation of specific CD4 T cells *in vivo* and *in vitro*, but different cytokine secretion patterns. Transgenic T cells stimulated with myeloid DC *in vivo* produced significantly higher levels of IL-4 and IL-10 compared to lymphoid DC-stimulated T cells which produced IFN-□; these cytokine secretion profile of activated T cells correlated with Th1/Th2 associated antibody isotypes (Pulendran et al., 1999). A similar system employing wild-type mice led to the same relationship (Maldonado-Lopez et al., 1999a and b). The differences in DC priming were largely attributed to IL-12 production by the DC, as lymphoid but not myeloid DC produced IL-12 when matured, but no differences in CD40, B7, or MHC II expression was observed (Reis

e Sousa et al., 1997). Similar results suggested different DC subsets were responsible for Th1 and Th2 priming in the human (Rissoan et al., 1999). Interestingly, in this study, although both CD40-L matured myeloid (DC 1) and 'lymphoid' (DC 2) subsets supported a strong allo-specific proliferation of naive T cells, the myeloid subset was found to be pro-Th1 in terms of T cell priming. Moreover, in the experiments of Maldonado-Lopez et al., mice receiving a 1:1 mixture of myeloid to lymphoid DC mounted a mixed response.

This raises a central question of how predominant Th1 or Th2 responses can be generated in an unmanipulated animal, if separate DC subsets are responsible for polarization, in situations in which antigen is presumably not restricted in its uptake by one particular APC type, and furthermore, cannot easily account for the finding that lymphoid DC cannot carry antigen from the periphery to a secondary lymphoid organ, without an accompanying model of DC-DC antigen transfer (Kalinski et al., 1997; Smith and Fazekas de St Groth, 1999; Carbone et al., 2004). Also, this type of Th1/Th2 control could represent a dangerous mechanism evolutionarily as it "may be restricted by its inherent rigidity", resembling selection more than adaptation (Vieira et al., 2000) and because some pathogens can effect the behavior of DC, creating "a potential risk for the immune system if it relies on the information provided by directly infected DC" (Kalinski et al., 1999). These concerns are restated in a recent review: "most pathogens should have no problem 'out-mutating' our germline-encoded recognition systems, in the same way as they rapidly become resistant to new antibiotics" (Kalinski and Moser, 2005). Finally, inherently polarizing APC subsets are difficult to reconcile with the substantial experimental evidence for

immune deviation, and, as discussed earlier, with the manner in which the Th1/Th2 phenotype evolves during the course of an immune response.

Important to keep in mind is the *in vitro* nature of many experimental systems employing both mouse and human derived DCs – in such systems, variables such as the ratio of plated DCs to responding T cells might dramatically influence the Th1/Th2 phenotype of the response generated (Tanaka et al., 2000). A further aspect of most experimental systems examining the role of DCs that should particularly be kept in mind is the use of various maturation/growth signals employed in preparatory steps, which are often needed to obtain sufficient numbers of dendritic cells. These procedures might well affect the behavior of DC both *in vitro* and *in vivo*. This statement attests to the complex biology of DC, and our still limited understanding of the interplay between elements of the innate and adaptive immune system. For example, human myeloid DC, which were initially reported as pro-Th1, have been found to support the generation of Th1, Th2, or Th0 responses *in vitro*, depending on reagents used in their maturation (Vieira et al., 2000). In another study, human monocyte-derived DC were found to mature into both pro-Th1 and pro-Th2 phenotypes, depending on exposure to differential microbial signals (de Jong et al., 2002). In the mouse, similar flexibility of the pro-Th1/Th2 polarizing capacity of DC subsets has been demonstrated employing bone marrow-derived DC, plasmacytoid DC (Boonstra et al., 2003), and isolated, purified splenic DC populations (Manickasingham et al., 2003). Furthermore, it has been demonstrated that much plasticity exists between DC precursors and their eventual surface marker-defined phenotype (Martin et al., 2000; Shortman and Wu, 2001; del Hoyo et al.,

2002; Shortman and Wu, 2004; reviewed in Pulendran, 2005), supporting the position that the Th1/Th2 inducing properties/signals are not intrinsic functions of any one APC population, though evidence suggests that there "might be restraints on DC flexibility" (Pulendran, 2005); this statement is supported by the finding that, while CD8+ DC generally bias towards the generation of Th1 effectors in "neutral" *in vitro* conditions, and CD8- DC towards Th2 effectors, these biases are "not very pronounced" and are easily swayed by culture conditions (Manickasingham et al., 2003).

The idea of *differently matured* DC subsets influencing Th1/Th2 development in response to certain infectious agents might represent a more physiologically relevant solution. In part this is because different subsets of DC can be characterized by the expression of different TLRs. For example, in the human, myeloid DC have been shown to express all ten TLRs except TLR 9, which responds to unmethylated CpG motifs, while plasmacytoid DC express TLR 9, in addition to TLR 1,6, and 7 (Mazzoni and Segel, 2004). Thus, DC subsets receiving activation signals through differentially regulated TLR might be critical in establishing pro-Th1/Th2 environments in the early phases of infection. The differential expression of TLR on different DC subsets might also be a more worthwhile tool for nomenclature than the current 'lymphoid/myeloid' scheme (Kadowaki et al., 2001).

Interestingly, while activation of DC through TLR causes release of cytokines and chemokines in a wide variety of experimental systems, several systems require CD40-L ligation for maximal DC effector activities, especially with regards to efficient CD4 and CD8 T cell priming and IL-12 production (Yasumi et

al., 2004, Krug et al., 2001, reviewed in O'Sulivan and Thomas, 2003). This leads to the suggestion that in some situations, effector CD4 T cells (Smith et al., 2004) or perhaps NKT cells, through recognition of their specific ligand on DC (Fujii et al., 2004) are required for full activation of, and therefore, the pro-Th1/Th2 potential of DCs. The implication of effector CD4 T cells in helping the generation of new helper cells, through the full activation of DC offers another interpretation of earlier studies finding pre-existing helper cells necessary for the generation of new, functional helper cells (Bretscher, 1986).

1.11.3 Variables affecting Th1/Th2 phenotype: costimulatory molecules

A related variable which has been studied extensively in Th1/Th2 differentiation is the role that different costimulatory molecules might play in polarizing responses. Several studies have implicated the costimulatory molecule B7.2 (CD86) as a pro-Th2 signal. Early studies employing B7.2 blocking antibodies (Kuchroo et al., 1995) and more recent *in vivo* and *in vitro* studies employing B7 or CD28 KO mice and different antigens (Zhang et al., 2004; Dong, 2003; Brown et al., 2002) clearly support the importance for B7.2 signaling and Th2 response phenotype. Importantly, the pro-Th2 activity of B7.2, a classical T-cell costimulator, need not represent a distinct Th2 signal, but could instead be a reflection of more efficient T cell interactions, facilitated by a B7.2 bearing APC, resulting in differential activation of T cells in these experimental systems because of a

difference in signal strength. This represents a different, quantitative interpretation of results, as compared to a purely qualitative one in which B7.2 signaling through CD28 is viewed as inherently Th2 promoting (Schweitzer et al., 1997).

The discovery of the inducible costimulatory molecule ICOS on TCR activated human T cells was immediately associated with Th2 responsiveness (Hutloff et al., 1999). Interestingly, ICOS upregulation on T cells was found to be induced by CD28 signaling, (McAdam et al., 2000) suggesting that the pro-Th2 activity of B7.2, described above, could be in part due to upregulation of ICOS (via a quantitative or qualitative model, as mentioned). Indeed, working with human cells in culture, it was demonstrated that blocking antibodies to B7.2, but not B7.1, impaired ICOS upregulation following a six day mixed lymphocyte reaction (Beier et al., 2000). While this result supports a B7.2-ICOS dependent circuit in polarizing Th2 responses, more recent evidence employing a variety of knockout mice has found that CD28-mediated upregulation of ICOS is not essential for normal ICOS function *in vivo* (Suh et al., 2004). A similar state of affairs exists with regards to OX40, another costimulatory molecule expressed on activated T cells, and its ligand, expressed on activated B cells, DC, and other tissues (reviewed in Croft, 2003). Again, while several studies support a role for OX40 signaling in the preferential development of Th2 responses, other evidence suggests that "differentiation is controlled by the level of expression of multiple accessory molecule pairs integrated with the number and affinity of peptide/MHC complexes" (Rogers and Croft, 2000). I believe this statement to be very relevant at a time when the increasingly complex

molecular events surrounding the primary activation of T cells and APC have yet to be described satisfactorily.

1.11.4 Variables affecting Th1/Th2 phenotype: nature of the MHC II binding ligand

The development of altered peptide ligands (APL), engineered analogs of immunogenic peptides known to be able to activate CD4 T cells, brought with it the question of whether the nature of an immunogenic peptide could impact the Th1/Th2 nature of the response generated by 'specific' CD4 T cells. In effect, this avenue of investigation centers largely on the role of signal one in determining the phenotype of the response generated, as compared with the role of signal two costimulators, as described in the previous subsection. Early *in vitro* studies demonstrated that certain APL brought about T cell anergy in comparison with proliferation by the wild-type peptide, and this correlated with unique phosphorylation events in the TCR-CD3 complex, suggesting a direct impact of signal one on T cell response phenotype (Sloan-Lancaster et al., 1994). It is important to keep in mind, when considering the Two Step Two Signal Model of T cell activation, that the nature of the peptide not only can impact the interaction of a naive CD4 T cell and an antigen-bearing APC (signal one), but also the interaction between an effector CD4 T cell and the same APC. The latter interaction reflects a critical step in the activation of a naive CD4 T cell, but is not mechanistically equivalent to 'signal one'.

Constant and Bottomly extensively review data from their *in vivo* and *in vitro* experiments showing a relationship between the affinity of one specific peptide for the MHC II in two different strains of mice and the Th1/Th2 CD4 T cell phenotype generated (Constant and Bottomly, 1997). In preliminary experiments, high peptide – MHC II affinity correlated with Th1 responses, while lower affinity correlated with Th2 responses, presumably because of stronger versus weaker T cell – APC interactions. Furthermore, APL generated from this peptide, which bound with either greater or less affinity, could alter the response phenotype as proposed by the relationship proposed in the last sentence.

These and similar experiments are interpreted as defining an important role for 'signal one' in generating either Th1/Th2 responses, and in a way echo the ideas in some early immune-deviation work, proposing that different pro-cell-mediated and pro-humoral elements, present in the nominal antigen, affected the response. A recent interpretation of similar APL data, using limiting dilution analysis, suggests that "qualitative effects of APLs ... may be explained by quantitative effects of Ag dose on the alteration of the cytokine milieu" (Grakoui et al., 1999).

1.11.5 Variables affecting Th1/Th2 phenotype: antigen dose

The impact of dose of antigen on immune response phenotype was analyzed extensively by Parish in the mid-1970s, as discussed earlier. These findings were extended *in vitro* (Bretscher, 1979, Bretscher, 1983). More recent studies have analyzed this relationship further, especially as some investigators have focused on

the possibility that any given vaccine, if incorrectly dosed, could potentially bias the immune response towards a non-protective pole. A clear example of this situation was presented in the murine leishmaniasis system where it was shown that the number (dose) of parasites used for challenge significantly shaped the phenotype of the immune response (Bretscher et al., 1992). This study demonstrated that in "innately susceptible" BALB/c mice, challenge with a small number of parasites induced a cell-mediated response that was able to protect animals against a subsequent normally lethal challenge. The protective state generated in these mice was demonstrated to be due to parasite-specific, IFN-γ producing T cells, as compared to predominant IL-4 production by T cells in mice challenged with only a high number of parasites (Menon and Bretscher, 1996). The same relationship between dose and the Th1/Th2 response generated was seen in mice challenged with BCG (Power et al., 1998) and with the noninfectious antigen SRBC (Ismail and Bretscher, 1999). Several other studies also correlate lower antigen dose with predominant Th1 responses and higher antigen dose with Th2 responses, using a variety of antigens and in a variety of strains and species, including humans (Guery et al., 1996; reviewed in Bretscher et al., 2001, Bretscher et al., 2002).

However, several other studies have correlated low antigen dose with Th2 responsiveness, and increasing antigen dose with predominant Th1 responses (reviewed in Constant and Bottomly, 1997; Grakoui et al., 1999). These studies (cited in Constant and Bottomly, 1997, Table 1) include experimental procedures in which protein antigen is continuously administered over a period of time, and *in vivo* and *in vitro* systems employing peptide as antigen and TCR transgenic CD4 T cells.

One potential reason for the different outcomes of dose in systems employing peptide as antigen, whether *in vivo* or *in vitro*, could be related to different APC populations (Constant et al., 1995) predominantly presenting either exogenous MHC II 'decorating' peptide or endogenously processed protein-derived MHC II peptides.

The correlation between peptide dose and Th phenotype generated is solid, and has been demonstrated in several experimental systems. However, as substantial and historical evidence supports the low dose-Th1 / high dose-Th2 relationship, especially employing *in vivo* experimental models in the context of infectious disease, vaccination strategies, and tumor therapy (Bretscher et al., 2002), I believe it more pertinent to work within this theoretical framework. However, it is conceivable that that the impact of peptide binding (APL-like effects) on the Th1/Th2 balance could augment the influence of antigen dose in certain situations.

1.11.6 Variables affecting Th1/Th2 phenotype: route of antigen exposure

If the route of antigen exposure influences the Th1/Th2 phenotype of the subsequent immune response, it would be an important variable in vaccine design and immunotherapy. The most logical impact that the route of exposure could have upon the immune response seems bound to involve elements of the body's innate defenses. As effects the ability of a given dose of antigen to generate either a Th1 or Th2 response in experimentally, evidence suggests that several different routes of immunization are similar, including i.v., s.c., and i.d., in that relatively high and low

doses of antigen respectively favor generation of Th1 and Th2 cells (Menon and Bretscher, 1998; Power et al., 1998). An exception is antigen exposure via airway epithelium in which "almost all models where T cell priming was successfully initiated...the outcome was predominance of Th2 responses" (Constant et al., 2002).

This trend is highlighted by the finding that intranasal delivery of *Leishmania major* to 'resistant' C57Bl/6 mice results in a predominant Th2 response (Constant et al., 2000) while challenge via most other routes results in a Th1 response (Menon and Bretscher, 1998). These experiments have been further analyzed, looking in particular at the mechanism of airway immune deviation. The resident APC, the cytokine environment, and cell trafficking were reported as factors which influenced the antigen-specific Th2 phenotype. In particular, a $CD11c^{bright}$ population of DC/Langerhans-like cell was reported to preferentially ingest both parasite and protein (OVA) antigen, produce significant amounts of IL-10 and IL-6, and the majority of these cells remained in the tissues instead of migrating to draining lymph nodes (Constant et al., 2002). This finding, and experiments done in manipulated animals lacking lymph nodes, in which responses were similar to those in wild-type animals suggests that local T cell priming in "tertiary" lymphoid organs might contribute to the Th2 response. A recent study has also implicated □□ T cells as critical in the development of pulmonary allergic inflammation (Zuany-Amorim et al., 1998). These studies highlight the unique pro-Th2 environment encountered in the airway epithelium. However, Th1 responses can be mediated in the lung, for example against mycobacteria, even in the presence of concurrent strong, helminth-induced, Th2 responses (Erb et al., 2002).

1.11.7 Variables affecting Th1/Th2 phenotype: the number of antigen-specific CD4 T cells

Several studies have found a relationship between the number of responding T cells and the Th1/Th2 response phenotype generated. With antigen concentration held constant, T cells supporting DTH responses were generated with low densities of spleen cells while T cells supporting antibody responses arose with higher densities (Bretscher, 1983a). The same dependency on the number of T cells responding and the phenotype generated was obtained *in vivo* using an adoptive transfer system (Bretscher, 1983b). Similar results have been reported in a system employing nude mice reconstituted with varying numbers of naive BALB/c spleen cells and challenged with *Leishmania* parasite. Reconstitution with high number of cells resulted in parasite susceptibility, associated with a Th2 response phenotype, while low numbers of cells resulted in a protective Th1 response (Powrie et al., 1994; Mitchell et al., 1981). In a related approach, BALB/c mice transiently depleted of a substantial number of CD 4 T are able to clear the parasite in a Th1-dependent manner (Heinzel and Rerko, 1999; Uzonna and Bretscher, 2001).

A more detailed study on the role of T cell numbers in determining immune response phenotype was carried out in an adoptive transfer system employing a non-infectious antigen (xenogeneic RBC). These experiments clearly demonstrated that more CD4+ cells used for reconstitution favored a Th2 response, as characterized by antigen-dependent IL-4 production by CD4 T cells, while IFN-□was produced when

animals were reconstituted with fewer cells and challenged with the same amount of antigen (Ismail and Bretscher, 1999). While these experimental systems are quite artificial, it is not difficult to imagine that within the CD4 T cell compartment of any given individual, different numbers of CD4 T cells will be specific for any given antigen, and that this number could vary greatly between individuals. Furthermore, the influence of the number of CD4 T cells is dependent on the type of antigen, as a more complex/larger antigen, generating more peptides, could potentially activate a larger number of CD4 T cells than a less complex/smaller antigen. This model fits Parish's observations on the impact of the degree of "foreignness" of an antigen and the type of response generated against it (Parish, 1971a; Ismail et al., 2005).

1.10.8 Variables affecting Th1/Th2 phenotype: CD4 T cell rate of division

There is considerable evidence supporting a relationship between the number of CD4 T cell divisions and cytokine secretion pattern. Detailed *in vitro* studies employing polyclonal anti-CD3 stimulation of sorted naive CD4 T cells have demonstrated that the secretion of critical Th1/Th2 defining cytokines, including IL-2, IL-4, IFN-γ, and IL-10, is dependent on the number of cell divisions the activated CD4 T cell undergoes (Gett and Hodgkin, 1999). In this study, while IL-2 production declined with progressive cell divisions, the production of IFN-γ and IL-4 increased. A trend for earlier production of IFN-γ than IL-4 was noted in this study; however, no predominant Th1 or Th2 phenotypes were observed in this system,

possibly due to conditions of culture, most notably, the need for exogenous IL-4 and IL-2 in priming cultures. A related study found conclusive evidence for the expression of IFN-γ before IL-4, which required T cells to pass through at least three cell cycles (Bird et al., 1998). Very interestingly, IL-10 production by stimulated T cells was only seen in cells having undergone extensive division, as determined by CFSE. These trends suggest a model of Th1/Th2 differentiation based on "functional strategies" employed by the immune system (Gett and Hodgkin, 1998). For example, naive T cells proliferate in response to antigen, secreting IL-2, then IFN-γ, if the antigen/pathogen is not cleared after a certain period of time/number of cell divisions by IFN-γ associated mechanisms (a Th1 response), allowing the continuing proliferation of specific T cells, then a new strategy is employed: a shift towards IL-4 production and a Th2 response.

Importantly, the absolute number of cell divisions is unlikely to soley determine a given cell's cytokine secretion profile in the view of long-lasting, strong Th1 responses. Instead, Salvin's early descriptions of the kinetics of the immune response, and several examples of progression from protective to chronic human disease states support a model more centered on the *rate* of division of CD4 T cells, perhaps during a critical window of time after activation, in determining the ultimate stable phenotype of the response (Salvin, 1958, Bretscher et al., 2001).

An important early regulator of T cell division, especially in *in vitro* systems, is IL-2. Hodgkin and colleagues have recently put forth a revised model of T cell proliferation that centers around concentration of IL-2 and T cell responses (Deenick et al., 2003). Experimentally, small changes in the concentration of IL-2 in culture

were found to have great impact on the number of cell divisions during a given period of time (the rate of division), and the total number of cells present, while changes in the amount of anti-CD3 (signal one/antigen) were found to impact the time required for responding T cells to enter their first division. These observations may be useful in interpreting a wide variety of data, especially when viewing *in vitro* systems, in which differences (perhaps very small differences) in the inital amount of IL-2 present, could lead to differences in response phenotype of responding cells (via cell division rate), say at day five. The *in vitro* data discussed earlier regarding APL (changes in the TCR-mediated signal one), and the differing phenotypes of T cells stimulated *in vitro* (perhaps with slightly differing concentrations of IL-2 or differing culture periods) might well be explained in part by incorporating some of Hodgkin's ideas and the notion of CD4 T cell division rate influencing Th1/Th2 phenotype. A further important point to keep in mind when analyzing data obtained from *in vitro* systems is that, at least in some laboratories criteria, "it is not possible to mimic the speed of the *in vivo* proliferative burst (of CD4 T cells) *in vitro*" (Fazekas de St. Groth et al., 2004). Thus, observations made *in vitro* regarding the rate of division might not accurately reflect *in vivo* kinetics.

It is difficult to reconcile the body of APL data and the predicted TCR-mediated effects on the Th1/Th2 phenotype, with observations that the amount of, or rate of, cell division can influence the cytokines produced by an activated cell, without incorporating other variables. One final note: if IL-2 does influence the number of times a given CD4 T cell divides, and the cytokines it produces, then it could be hypothesized that the greater the number of CD4 T cells activated initially

(in response to antigen recognized on a single APC, or tight cluster of APC) the more IL-2 will be available in the microenvironment, causing more cell division and progression towards a Th2 response phenotype. This scheme suggests a possible relationship between the rate of cell division, the number of responding CD4 T cells (1.10.7) and the antigen dose (1.10.5).

1.11.9 Variables affecting Th1/Th2 phenotype: antigen-specific regulatory CD4 T cells

In a relatively short period of time, an abundant and complex literature has accumulated describing T regulatory cell immunobiology. With regards to the initial Th1/Th2 differentiation of naive CD4 T cells, some more recent studies have found an impact of antigen-specific regulatory T cells. This research has centered on immune responses generated against infectious agents, suggesting that in certain situations, "a distinct subtype or activation status of APCs exists, which promotes the differentiation of regulatory rather than effector T cells from naive precursors" (McGuirk and Mills, 2002). Experimentally, these regulatory cells have been characterized as 'Tr1' or 'Th3' cells by virtue of their IL-10 and TGF production, and their *in vitro* suppressive effects and growth patterns.

Specifically, antigen-specific CD4 T cells, producing predominantly IL-10, were shown to be generated both *in vivo* and *in vitro* in response to challenge with *Bordetella pertussis* (McGuirk et al., 2002). These cells were shown to be capable of inhibiting a protective Th1 response while not appreciably affecting a non-protective

Th2 response. A similar role for pathogen-specific IL-10 producing regulatory cells has been suggested in patients with chronic pulmonary tuberculosis (Delgado et al., 2002) and chronic suffers of hepatitis C (MacDonald et al., 2002).

While the induction of pathogen-specific regulatory T cells might be important in defining the Th1/Th2 phenotype in some clinical instances, it is difficult to imagine a role for antigen-specific regulatory T cells in non-infectious situations. One caveat to this position is the conceivable possibility that a pre-existing CD25+ CD4 suppressor T cell, generated in the thymus and specific for a self antigen (for a recent review, see O'Garra and Vieira, 2004) could cross-react with a foreign determinant, thus exerting an effect on the Th1/Th2 phenotype of a primary immune response generated against such an antigen. Older observations also suggest that antigen-specific CD4 T cells cells activated under conditions where humoral (Th2) immunity is induced can suppress the generation of DTH responses against the same antigen (Ramshaw et al., 1976; Ramshaw et al., 1977).

1.10.10 Variables affecting Th1/Th2 phenotype: adjuvants

Adjuvants have long been known to affect the phenotype of the immune response, with 'complete' formulations containing mycobacterial products, such as complete Freund's adjuvant, and the aluminum salt based adjuvant alum representing prototypical Th1 and Th2 enhancing adjuvants respectively (although in several situations, CFA enhances antibody responses as well). Several other chemical and bacterial products have also been employed to enhance either Th1 or Th2 responses

(or both) in a variety of experiments and vaccine trials (reviewed in Nossal, 1999). Since this thesis is more concerned with variables of immunization which can be manipulated to be either Th1 or Th2 promoting in the absense of adjuvant, and since the mechanisms by which some adjuvants exert their effect is not well understood, I will not discuss adjuvants further. However, I will note that the use of certain cytokines, especially IL-12, in biasing responses towards a Th1 pole, is synonymous with the use of adjuvant, since many believe that production of 'innate cytokines' represents, at least in part, the manifestation of some of the effects of the adjuvants.

1.11.11 Variables affecting Th1/Th2 phenotype: general systemic variables influencing immune response phenotype

Obviously, the genetic background of an individual can have a significant impact on the type of response generated against a particular antigen; experimentally, this is perhaps best exemplified by the responses generated by BALB/c and C57Bl/6 mice when challenged with the same number of *Leishmania* parasites. It is also worthwhile to mention that, regarding replicating organisms, genetic differences between strains of a given virus, parasite, or bacteria can impact the Th1/Th2 phenotype of the response. For example, differential replication rates between any two strains of a bacteria could result in separate, strain-specific Th1 and Th2 responses upon challenge with the same number of each organism because of differences in *effective* dose – a product of the initial inoculum and the differential capacity to increase that initial dose by replication (North and Izzo, 1993).

It is well known that in certain physiological situations, pregnancy for example, the Th1/Th2 balance of the immune system seems to be powerfully influenced by a circuit of non-immunological, hormonal signals (Seder and Mosmann, 1999). The exciting field of neuroimmunology has the potential to uncover several similar and more subtle influences on the immune system by non-immune stimuli; a recent study with human volunteers has made a correlation between better (more) sleep and a shift towards Th1 responsiveness (Dimitrov et al., 2004). Seasonal changes, especially changes in daylight hours, are also hypothesized to influence some individual's overall Th1/Th2 balance (Lam et al., 2004). Finally, exercise, general health and nutritional variables, especially the influence of certain vitamins including vitamin A, and certain environmental pollutants, have been found to influence not only the general state of the immune system, but also the pro-Th1/Th2 balance of the immune system (Malm, 2004; Stephensen, 2001; Van Zijverden and Granum, 2000).

1.11.12 Variables affecting Th1/Th2 phenotype: concomitant activation of non-CD4 T cell regulatory populations

It is important to mention that the activation of cells other than CD4 T cells during the early stages of an immune response (whether CD4 T cell-dependent or not) can potentially result in important regulatory populations influencing the Th1/Th2 phenotype of the response. For example, as discussed in 1.9.1, CD8 T cells activated under conditions generating predominant cell-mediated responses against a

given antigen have been found to actively suppress the generation of humoral (Th2) responses against the same antigen when adoptively transferred into an animal challenged in a manner known to generate humoral responses (Ramshaw et al., 1977). This concomitant activation of a non-CD4 T cell, phenotype-regulating population represents not only a possible mechanism whereby long-term, predominantly cell-mediated Th1 responses can be *locked-in*, but could potentially be an important factor in the initial generation of a stable Th1/Th2 phenotype. The establishment of antigen-dependent cellular networks consisting of CD4 and CD8 T cells, responsible for antigen-specific immune class regulation (Bretscher, 1981; Bretscher, 1983; Tuttosi, 1993) represents a related, but more complicated and perhaps more complete mechanism of control over the cellular/humoral phenotype of a response than the more simplistic model of cross-regulation concentrating only on polarized CD4 T cells and their products (see 1.11.1).

This type of system has recently been proposed in the establishment of some protective Th1 responses in murine leishmaniasis (Uzonna et al., 2004). In this report, concomitant activation of Leishmania-specific CD8 T cells was required to establish a protective Th1 response. Further analysis revealed that these CD8 T cells were required to modulate parasite-specific CD4 T cell responses rather than respond directly against the parasite. An intriguing possibility is that IFN-□ production by CD8 T cells, coordinately induced with Th1 CD4 T cells in certain situations, is a prime factor in favoring development and lock-in of Th1 responses while inhibiting the generation of and or proliferation of Th2 cells (see 1.11.1).

Thus, *qualitative* differences in antigen/pathogen challenge (influencing the amount of MHC I expressed peptides, for example), could allow for differential activation of non-CD4 T cell populations with the potential to strongly influence the ultimate Th1/Th2 phenotype of the response generated. It is therefore possible that *quantitative* differences in the number of CD8 T cells activated, either because of dose, the number of antigen-specific precursor CD8 T cells, or because of qualitative differences between antigen, represent an important variable in establishing and regulating the Th1/Th2 phenotype of antigen-specific immune responses.

1.12 Stability of the Th1/Th2 phenotype once acquired

1.12.1 Functional evidence

As discussed earlier, the initial reports of long-term Th1/Th2 murine clones mentioned their phenotypic stability over several months, with the exception of IL-2 production, which was gradually lost amongst some Th1 clones (Mosmann et al., 1986). Further studies showed that shorter-term Th0 clones tended to gradually adopt a more polarized Th1 or Th2 phenotype with longer culture periods (Firestein et al., 1989), and that all Th phenotypes could be generated from initial IL-2 producing precursor CD4 T cells (Swain et al., 1988, Sad and Mossman, 1994).

Thus, individual cells, or perhaps clonal populations of cells, pass through a period of some phenotypic plasticity with regards to cytokine production (Th0), which is lost after longer periods of time, or after a certain number of cell divisions,

or after more intense polarizing stimuli. Stable, long-term Th1 and Th2 clones seem to hold their phenotype long-term both *in vitro* (Mosmann et al., 1986, Wang and Mosmann, 2001) or when transferred *in vivo* (Swain, 1994). This paper provided direct evidence that memory CD4 T cells could be derived from an effector population of antigen-specific CD4 T cells, and that long-lived memory cells tend to retain the Th1/Th2 phenotype of effector cells they derived from (Swain, 2003). Under extreme deviating conditions *in vitro* (exogenous cytokines), it has been shown that differentiated Th1 cells can be influenced by the presence of IL-4 to change their phenotype to Th2; no effect of IL-12 on Th2 cells was observed (Perez et al., 1995). Such situations are not likely to be experienced *in vivo*, except perhaps in situations of overwhelming infection. Indeed, in experimental models with mice infected with a large number of helminth parasites, it has been shown that strong Th2 responses can down-regulate Th1 responses, both in terms of characteristic cytokine production (IFN-□) and their protective potency (vaccinia-specific CTL), suggesting that in some situations a polarized and dominant Th2 response can sway the systemic phenotypic balance (Pearce et al., 1991; Actor et al., 1993, Liesenfeld et al., 2004). Together, these observations support the view that an altered, systemic Th1/Th2 balance most often reflects a pathological consequence of infection, and not a mechanism of immune regulation.

Further evidence of long-term memory Th1 and Th2 cells is seen in experimental systems in which animals primed to generate Th1 cells respond with the same phenotype to a later, normally lethal Th2 inducing challenge (Bretscher et al., 1992). In fact, successful vaccination, with long-term memory, is often

presumably dependent on the continual presence of a population of properly Th1 or Th2 'deviated' CD4 T cells; their maintenance, and the variables influencing the generation of antigen-specific memory cells is outside the scope of this thesis.

1.12.2 Molecular evidence

Studies of newly activated and differentiated CD4 T cells at the molecular level support the conclusions briefly discussed above, that Th1/Th2 phenotype is a relatively stable acquisition. Many aspects of the intracellular signaling pathways involved in T helper cell lineage commitment have been characterized in the past fifteen years or so. Specifically, certain MAP kinase pathways, which are activated in response to a variety of extracellular/intracellular stimuli through upstream elements, have been found to be specifically involved in signaling through Th1-associated cytokine receptors, such as IFN-γ, IL-12 and IL-18 (reviewed in Szabo et al., 2003).

The differential activation of upstream signaling pathways, such as those that control MAP kinase-dependent pathways, lead to further downstream events, such as the activation and nuclear translocation of unique STAT signallers. For example, signaling through the IFN-γ receptor leads to the activation of STAT1, and mice deficient for STAT1, IFN-γ receptor, or IFN-γ, and humans with defective IFN-γ signalling, all feature significantly impaired immune responses to mycobacteria and some viruses; similar evidence has been shown for the IL-12 pathway, signaling through STAT4 (Szabo et al., 2003). These data support the conclusions discussed

earlier demonstrating an important role for IFN-γ and IL-12 in the development of Th1 responses. Similarly, IL-4 signaling has been shown to proceed through a STAT6-dependent pathway (Ouyang et al., 1998). The importance of STAT-dependent signalling has been demonstrated recently in experiments utilizing adoptive transfer of transgenic CD4 T cells on either a STAT4 or STAT6-deficient background into syngeneic STAT4 or STAT6 hosts. Upon immunization, transferred transgenic cells responded predominantly with the predicted phenotype (STAT4-deficient – Th2; STAT6-deficient – Th1), regardless of the pro-Th1 or pro-Th2 environment into which they were transferred (Chitnis et al., 2004), reflecting the general inability of polarizing cytokine miliea to significantly influence CD4 T cell development through non-STAT dependent pathwways.

The elucidation of factor-specific signaling pathways has led to the description of several pro-Th1 and pro-Th2 transcription factors which are ultimately involved in the phenotypic differentiation of naive CD4 T cells. In fact, these transcription factors are often viewed as 'master switches' controlling Th1/Th2 development. However, this simplistic view, of 'master switches' being turned on and off in determining the Th1 or Th2 phenotype of CD4 T cells, is undoubtedly complex beyond our current knowledge, particularly in view of Kelso's groups' data suggesting extreme effector heterogeneity at the single-cell level in controlled pro-Th1 and pro-Th2 *in vitro* cultures. However, recent work on Th1 and Th2 'master switches' has offer insights into the relative stability of polarized CD4 T cells.

The transcription factor T-bet was shown to be selectively induced in developing Th1 cells, leading to the production of IFN-γ (Szabo et al., 2000),

through a STAT1 dependent and not STAT4 (IL-12) dependent pathway. It was further shown that T-bet expression not only led to IFN-γ production, but repressed IL-4 and IL-5 production, even when T-bet was expressed in polarized Th2 cells. Thus, T-bet induction seems to cause a cascade of polarizing Th1 events, while repressing the development of pro-Th2 programs. This pro-Th1 program and T-bet's expression itself is heavily dependent on IFN-γ (STAT1), but once expressed, T-bet can cause upregulation of the IL-12 receptor, leading to further influences of IL-12 as a Th1 polarizing condition (Afkarian et al., 2002).

Similar to T-bet's role in Th1 polarization, the transcription factor GATA-3 was found to be preferentially expressed in Th2 cells, leading to the upregulation of Th-2 associated cytokines IL-5 and to a lesser extent IL-4 (Zheng and Flavell, 1997). Furthermore, GATA-3 expression was found to inhibit Th1 cell differentiation (Ouyang et al., 1998), in part due to the down-regulation of IL-12 receptor expression. Thus, T-bet and GATA-3 (in addition to others) represent the major 'endpoints' of eary pro-Th1/Th2 signaling pathways involved in phenotype development. In contrast to Zheng and Flavell's interpretation that GATA-3 expression was "necessary and sufficient for Th2 cytokine expression" the targeted expression of GATA-3 in developing CD4 T cells exposed to either pro-Th1, pro-Th2, or neutral culture conditions (via cytokine environment), while inducing IL-5 production to levels similar to polarized Th2 clones, only led to sub-optimal IL-4 production, leading the authors to conclude that, "GATA-3 [expression] is permissive, but not sufficient, for full IL-4 enhancement", implying important roles

for other pro-Th2 transcription factors in the regulation of the arch-Th2-polarizing cytokine, IL-4 (Ranganath et al., 1998b).

T-bet and GATA-3 expression appear to be mutually exclusive in the early stages of CD4 T cell differentiation, under relatively extreme polarizing conditions *in vitro* (Rengarajan et al., 2000). The antagonistic effects of these transcription factors on the opposing phenotype suggests that, once in place, the Th1 and Th2 differentiation programs initiated by these factors is relatively stable. In fact, in the initial T-bet study, it was found that the effects of T-bet expression in more polarized Th2 clones were much less than when 'younger', presumably less-differentiated clones were used (Szabo et al., 2000). Very recent evidence supports a model in which an early event in Th1 polarization of CD4 T cells is the physical binding of GATA-3 by T-bet in the nucleus, facilitated by tyrosine phosphorylation of T-bet, thereby sequestering GATA-3 away from specific binding sites in the Th2 cytokine locus (Hwang et al., 2005). This offers an explanation as to how T-bet is able to upregulate expression of Th1-associated proteins while also repressing the expression of important Th2-associated genes. Together, molecular and functional evidence suggests that the final Th1/Th2 phenotype adopted by a CD4 T cell is relatively stable, as reflected by differential chromatin remodeling brought about by the activation of separate pro-Th1 and pro-Th2 transcription factors.

1.12.3 Th1/Th2-specific cell-surface markers

A sort of 'holy grail' in the investigation of Th1/Th2 subsets has been the quest to define subset-specific surface molecules, which could be used instead of or in addition to cytokine profile as reliable markers *in vivo*. Unfortunately, this effort has not yet led to many successes. One approach to defining distinguishing cell surface markers has been the investigation of chemokine receptors expressed by polarized CD4 T cells. Chemokines are involved in leukocyte recruitment, and therefore, based on the different effector functions of committed CD4 T cells, it is "perhaps not surprising that a rather striking pattern of expression of chemokine receptors of Th1 and Th2 subsets is emerging" (O'Garra et al., 1998). Thus, studies have found associations between Th1 cells and CXCR3 and CCR5, and between Th2 cells and expression of CCR3, CCR4, and CCR8 (reviewed in O'Garra et al., 1998).

Importantly, the expression of several chemokine receptors is dependent on activation status, and therefore is variable, and several other cell types (monocytes, neutrophils, DC, NK cells, B cells, eosinophils, and basophils) also can express some or all of these molecules (Cosmi et al., 2001). Therefore, while the expression of Th1/Th2-associated chemokine receptors can be of value in certain situations, it is not definitive criteria to differentiate CD4 T cell subsets, nor to assess their stability, especially *in vivo*. More recently, CRTH2, a molecule on human T cells was described as being preferentially expressed on Th2 and Tc2 cells with an activated phenotype (but also on basophils and eosinophils) (Tsuda et al., 2000). This marker has been found a more reliable indicator of Th2 cells *in vivo* (Cosmi, et al., 2000).

Earlier experiments proposed that CD26 and CD30 expression could potentially be used to differentiate polarized CD4 T effector cells *in vivo*. In the human, expression of CD26 on T cells was reported to correlate with the presence of Th1 cells. While this correlation was observed in cases of tuberculoid versus lepromatous leprosy (Seitzer et al., 1998) it has not been found in other clinical situations (Rogala et al., 2002). Similarly, CD30 expression on human CD4 T cells was found to correlate with IL-4 production and Th2 polarization, and furthermore, CD30 engagement by specific antibody was found to enhance IL-4 production from Th2 and Th0 clones, suggesting a role for CD30-dependent signaling in Th2 polarization (Del Prete et al., 1995 a and b). Murine CD30 has also been characterized (Bowen et al., 1996) and its role appears to be similar to that of CD30 in the human (Shimozato et al., 1999). Importantly, while CD30 appears to be a valuable tool in diagnosing the Th1/Th2 balance of an immune response, its expression on CD4 T cells is "highly dependent" on the presence of sufficient IL-4 (Cosmi et al., 2001), suggesting that some temporal variations in CD30 might occur during and after a specific immune response. This position is supported by an earlier study, which screened peripheral blood and human T cell clones, finding no evidence for unique CD30 expression on Th2 cells (Hamann et al., 1996).

Research has shown that Th1 effectors are in general more prone to undergo Fas/FasL-mediated activation induced cell death than polarized Th2 effectors. While early reports employed T cell clones or long-term cultures, the same pattern has been observed in more physiologically relevant short-term cultures (Zhang et al., 1997). A recent study has correlated the increased survival of Th2 cells with surface

expression of CTLA-4 (CD 152) (Pandiyan et al., 2004). While differences were noted between populations of polarized effectors at certain time-points of culture, expression of CTLA-4 *within* populations of polarized cells varied greatly as well, implying that expression of CTLA-4 is not a definitive surface marker of Th2-polarized CD4 T cells. These experiments analyzed TCR transgenic T cells differentiated into Th1 or Th2 effector cells *in vitro* via the addition of polarizing cytokines, leaving the *in vivo* relevance of the finding to be questioned. In conclusion, the typing and longitudinal tracking of Th1 versus Th2 cells via surface markers alone seems at present far from optimal.

1.13 Summary

By presenting the introduction in two general parts, one outlining the development of models of CD4 T cell activation, and the other summarizing variables found to influence immune deviation and Th1/Th2 phenotype development, I hope to have laid solid foundations for discussing my experimental hypothesis, and later, for discussing my experimental data and its implications.

2.0 Rational for experimental approach

2.1 The Threshold Hypothesis

Based upon the Two Step Two Signal Model of CD4 T cell activation, the Threshold Hypothesis describes a decision criterion by which the immune system can selectively respond against a specific challenge with either a predominant Th1 or Th2 response (Bretscher, 1991; Bretscher 1999). Unlike several related qualitative models of Th1/Th2 differentiation, for example, those based on differential expression of costimulatory molecules, perhaps induced by antigen recognition by separate APC, the Threshold Hypothesis is largely quantitative. The Threshold hypothesis grew out of an earlier "theory of immune class regulation", formulated before the discovery of Th1/Th2 cells and their various precursor states (Bretscher, 1974, Bretscher, 1981). In the modern formulation of the Threshold Hypothesis, replacing the earlier model's single variable of "inductive complexes" – a combined variable depending on the amount of antigen and the amount of 'help' present, the two prime variables envisioned to direct the immune response towards a predominant Th1 or Th2 pole are "the number of specific precursor helper T cells

available and the efficiency with which they are induced by antigen" with this efficiency being "antigen-concentration-dependent" (Bretscher, 1991).

Mechanistically, the variables of antigen concentration, or dose, and the precursor CD4 T cell frequency are envisioned to cause the differential expression of a "signal two" costimulatory molecule by an antigen-bearing APC. Optimal signal two expression is generated in the presence of optimal (or a relatively high numbers) antigen-mediated CD4 T cell – APC interactions, while fewer of these interactions leads to lower, sub-optimal expression of signal two. Optimal delivery of signal two to an antigen-specific CD4 T cell, above a certain threshold, results in the a promotion of a Th2 differentiation pathway, while a lower level of signal two results in predominant Th1 effector commitment (Bretscher, 1991). This model also incorporates (or better said, this model is based upon) a mechanism of self/non-self discrimination, as the few anti-self precursor CD4 T cells specific for any given self antigen would not usually result in the cooperative induction of sufficient signal two, above a critical threshold, and therefore would not be activated, but instead would be functionally deleted. A wealth of earlier observations, including those of Salvin, Pearson and Raffel, and Parish (see 1.8.2, 1.8.4, and 1.8.5) can be described much more easily in terms of the Threshold Hypothesis than by other models of Th1/Th2 polarization centering around specialized APC, the strength of MHC-TCR interaction, or regulatory T cells.

As discussed earlier, several more recent observations, both *in vivo* and *in vitro* support the Threshold Hypothesis and its emphasis on antigen dose and the number of responding CD4 T cells (see 1.10.5 and 1.10.7). Furthermore, the initial

cellular interactions envisioned by the Threshold Hypothesis to initiate Th1/Th2 differentiation might well be reflected by augmenting down-stream variables, such as cytokine microenvironments (1.10.1) and costimulatory ligand –receptor profiles on fully activated cells (1.10.3), which might be critical in maintaining or 'honing' phenotype. Importantly, the Threshold Hypothesis is satisfying teleologically; an antigen which presents the immune system with few foreign determinants, against which antibody would perhaps not be effective due to a low density of surface determinants, would be expected to generate a predominant Th1 response because of the interaction of few specific CD4 T cells, while challenge with a similar dose of more foreign antigen, against which antibody could be more effective, would be expected to preferentially generate a Th2-type response by virtue of more antigen-mediated CD4 T cell – CD4 T cell inductive events. Control of Th1/Th2 phenotype as described in this model is also appealing from an evolutionary perspective, as a system based solely on a panel of conserved, germline Th1/Th2-polarizing pathogen associated molecular pattern receptors, might be subverted by pathogen mutation, though Janeway argued against this concern (Janeway, 1989).

Finally, the Threshold Hypothesis accounts for the independence of concurrent non-crossreacting, predominant Th1 and Th2 responses (Ismail and Bretscher 1999; Erb et al., 2002). This model, with its spatially separated Th1/Th2 decisions made by separate populations of antigen-specific interacting lymphocytes, can more easily preserve the independence of concurrent responses than other models centered around professional APC able to simultaneously present determinants derived from separate antigens (Cervi et al., 2004), or ascribing

importance to the general cytokine milieu. A more complete discussion of how independence of immune responses is envisioned to be maintained by the Threshold Hypothesis is needed, and I will return to this point in the general discussion of this thesis, as it relates to experimental data that will be presented.

3.0 Research Objectives

The experiments reported here were conducted in order to investigate some aspects of the mechanism whereby antigen-mediated CD4 T cell cooperation can affect the Th1/Th2 phenotype of a primary, antigen-specific immune response:

(i) To determine if CD4 T cells specific for one determinant (peptide) of an antigen can affect the Th1/Th2 phenotype of responses generated by CD4 T cells specific for linked determinants of the same antigen, and to determine whether the Th1/Th2 phenotype of such diverse (but antigen-specific) responses are coherently regulated.

(ii) To test the prediction, made by the Threshold Hypothesis, that increasing numbers of antigen-specific CD4 T cells, in the presence of a limiting amount of antigen, can lead first to the generation of an antigen-specific Th1 response, and with increasing number, support the generation of an antigen-specific response with a strong Th2 component.

(iii) To investigate aspects of the mechanism of cooperation between antigen-specific CD4 T cells influencing the Th1/Th2 phenotype of the responses generated when the immune system is challenged concurrently with two non-cross-reacting antigens.

(iv) To critically investigate whether naive CD4 T cells (thymocytes), specific for one determinant of an antigen, can influence the Th1/Th2 phenotype of CD4 T cells specific for other linked determinants.

4.0 Materials and Methods

4.1 Mice

BALB/c (H-2^d) mice were obtained either from the animal colony of the College of Medicine, University of Saskatchewan, or from Charles River (Montreal, Quebec, Canada). DO11.10 mice (H-2^d), mice on a BALB/c background, expressing a transgenic MHC II-restricted TCR specific for OVA, were obtained from the animal colony of the College of Medicine, where they were bred from an original breeding pair purchased from Jackson Laboratories (Bar Harbor, Maine, USA).

All mice were housed under specific pathogen-free conditions. Routine screening was done to ascertain that mice were free of subclinical viral and bacterial infections. Mice employed for experiments, both naive cell donor mice (for adoptive transfer reconstitutions and for ELISPOT feeder cells) and lethally irradiated adoptive transfer hosts, were female and usually aged between 5-10 weeks. When a source of thymocytes was necessary, younger mice were employed, as thymocyte yields were much higher in younger mice (4-6 weeks). Mice obtained from off-campus sources were rested at least one week before use. All experiments were performed with ethical approval from the Canadian Council of Animal Care.

4.2 Antigens

Suspensions of SRBC in Alsever's solution were obtained from the Clinical Microbiology Laboratory, University of Saskatchewan. Sterility of fresh SRBC stocks was routinely assessed by the staff of the Clinical Microbiology Laboratory. Fresh suspensions of SRBC were used for each experiment, with samples of a given suspension serving as both the immunizing and recall (in the ELISPOT assay) antigen. SRBC obtained from the Clinical Microbiology Laboratory was never used past the expiry date provided by the supplier. SRBC stocks were stored in the suppliers storage media (Alsever's solution).

Suspensions of CRBC were obtained from the Poultry Center, University of Saskatchewan, from dedicated donor birds. CRBC were stored in Alsever's solution and their sterility assessed by incubation on rich media plates at 37ºC. Fresh suspensions of CRBC were used for each experiment – blood from an individual chicken was used within experiments as both immunizing and recall antigen. All XRBC were washed at least three times in sterile PBS before their further use in experiments. CRBC were stored for a maximum of three weeks.

Crystalized, lyophilized ovalbumin (OVA) (grade V) was purchased from Sigma Laboratories (St. Louis, Missouri, USA). Fresh preparations of OVA were solubilized in sterile PBS (10 □g/mL) at 4ºC by gentle rocking for at least one hour, or without rocking for at least 3 hours, and were then filter-sterilized through a 0.22

□m filter (Millipore, Molsheim, France). Fresh preparations of filter-sterilized OVA in PBS were employed in each experiment.

4.3 Media

Leibovitz media and RPMI 1640 supplemented with L-glutamine media were prepared from powdered stocks as per the manufacturer's instructions (Gibco Laboratories, Grand Island, New York, USA). Fresh solutions of media were sterilized by filtration through 0.22 □m filter (Millipore) and their sterility assessed by overnight incubation of filtered stocks at 37 ºC.

Complete RPMI media was prepared by further supplementing sterile RPMI 1640 media supplemented with L-glutamine with 10% fetal calf serum, penicillin-streptomycin (100 U/mL), $5x10^{-5}$ M □-mercaptoethanol, and 0.8% sodium pyruvate (100 mM).

Alsever's solution (pH 6.1), for the longer-term storage of XRBC, was prepared by combining dextrose (20.5 g/L), sodium citrate (8.0 g/L), citric acid (0.55 g/L), and sodium chloride (4.2 g/L) in distilled water. Fresh preparations of Alsever's solution were autoclaved for sterility.

Phosphate-buferred saline (PBS) was prepared from a stock solution (20x, pH ~7) containing 160g/L NaCl, 4g/L KCl, 23g/L Na_2PO_4, and 4g/L KH_2 PO_4 in deionized distilled (dd) H_20. Fresh preparations of 1x PBS were autoclaved for sterility. PBS containing 0.05% Tween 20 (PBST) was used extensively in the washing and development steps of the ELISPOT assay (as described in 4.7).

4.4 XRBC – protein coupling

OVA was coupled to XRBC by exposure to a tannic acid solution. A 5% solution of thoroughly washed XRBC in sterile PBS was incubated with an equal volume of a 0.005% solution of tannic acid in PBS for 20 minutes at 37 ºC, mixing every five minutes. Tannic acid treated RBC were then washed once in PBS (centrifuged for 10 minutes at 280–x g), resuspended to 5% solution in PBS, and then incubated with 200 μg of OVA (at 10 mg/mL) for a further 20 minutes at 37ºC, mixing every five minutes. After 4-6 further washes in sterile PBS, suspensions used for immunization were made by dilution of a specific volume of packed XRBC in PBS or sometimes in Leibovitz media.

4.5 Adoptive transfer and immunization

Naive BALB/c mice were lethally irradiated with 750-850 R using a ^{60}Co source (Gammacell 220, Atomic Energy of Canada Limitd, Ottawa). These irradiated mice were both reconstituted with naive donor cells, and when appropriate, immunized with antigen, on the same day of irradiation, or one day after irradiation, by a single i.v. injection (200 – 400 μL).

4.6 Preparation of single cell suspensions

Mice were sacrificed by cervical dislocation. Their spleens were aseptically removed and placed in 10 mL of cold Leibovitz media. Single cell suspensions were then prepared by gentle disruption of the spleen and forced passage through a sterile stainless steel mesh, employing a sterilized blunt glass rod, gently. These suspensions were then left for 1-3 minutes, allowing larger cellular aggregates and other debris to settle out of suspension. The supernatants from these 'de-clumped' suspensions were then collected (by leaving the final 0.5 mL behind) and washed twice in Leibovitz media by centrifuging at 280-x g for 10 minutes. Splenocytes were then resuspended in 2-10 mL of complete RPMI media (depending on whether spleens were obtained from irradiated, reconstituted mice (2mL) or from naive (10mL) mice). Sometimes, before counting spleen cells, a brief (10-15 seconds), second 'de-clumping' step was done to remove larger aggregates, especially when preparing immunized (as compared to naive) mice.

Viable leukocytes were enumerated via trypan blue exclusion using a haemocytometer. Usually, the average of two separate counts was taken to calculate the number of viable cells present. Throughout this process, cells and media were kept on ice whenever possible.

Thymocytes, used in some experiments as an alternate reconstituting population in adoptive transfer experiments, were prepared in exactly the same manner described above for splenocytes, with the exception that a 'de-clumping' step was rarely needed.

4.7 ELISPOT assay for the enumeration of cells producing antigen-dependent IFN-γ or IL-4

Antigen-dependent cytokine production was assessed employing an optimized ELISPOT assay (Power et al., 1999). For each experiment, ninety-six-well Unifilter nitrocellulose flat-bottom plates (Whatman-Polyfiltronics, Clifton, New Jersy, USA) used for the assay were prepared beforehand (1-5 days) by coating with purified antibodies specific for cytokines of interest. Specifically, each well was coated with100 μL of a 1 M bicarbonate buffer (pH 9.6) containing purified anti-IFN-γ (R4-6A2), or anti-IL-4 (11B11) monoclonal antibodies (Pharmingen, San Diego, California, USA) at a concentration of 1.25 μg/mL. Coated plates were stored at 4 ºC until their experimental use. Before their use in the ELISPOT assay – that is, before the addition of cells and antigen, coated plates were washed once with 200 μL/well of RPMI 1640 media and subsequently blocked with 200 μL/well of complete RPMI media for at least three hours at 37 ºC.

After removing the blocking media from ELISPOT plates, spleen cells (prepared as described in 4.6) from experimental mice were added to individual wells in a standard volume (generally 100 μL/well) but from stocks at various concentrations (Generally at $5x10^6$ cells/mL, but sometimes from $2.5x10^6$ – $1.5x10^7$ cells/mL). Preliminary experiments ensured that, as has been previously reported for this optimized ELISPOT assay employing XRBC as antigen (Ismail, 2000), the number of antigen-dependent IFN-γ and IL-4 spots observed is directly proportional

to the number of immune cells plated, as long as the total number of cells per well is brought up to $1.5x10^6$; when fewer than this number of immune cells was plated, spleen cells from a naive animal were added to wells using the formula: *number of naive feeder cells per well = $1.5x10^6$ cells per well – number of immune cells per well*. Generally, these naive 'feeder' cells were irradiated (1500 R of γ-irradiation from a ^{60}C source) before their use in the ELISPOT assay.

To accurately assess the number of antigen-dependent cytokine-producing cells (spots), at least three wells were plated with experimental and (when necessary) feeder spleen cells in both the presence and absence of antigen. Either 10 μL of a 10% XRBC solution or 2.4 μg of soluble OVA was added per well to create antigen-positive conditions in wells. The average difference of spots seen in antigen-positive wells versus antigen-negative wells was recorded as the raw number of antigen-dependent spots per the plated number of immune spleen cells. This value was then used to calculate the 'normalized response' (see 5.2.1). Importantly, in each experiment, irradiated feeder cells were plated alone in the presence and absence of antigen; generally, feeder cells themselves generated very few (1-3/$1x10^6$ cells) IFN-γ spots and sometimes slightly higher numbers (10-15/$1x10^6$ cells) of IL-4 spots – importantly, these spots were never antigen-dependent.

Fully loaded ELISPOT plates (containing immune cells, feeder cells, and antigen) were incubated at 37 ºC in the presence of 5% CO_2 for 8-10 hours, but sometimes up to 18 hours. Wells were then washed twice with PBST. To ensure that no residual cytokine production or cellular components persisted into further steps of the assay, wells were then washed twice with ddH_2O, waiting ~5 minutes

between washes, to lyse any remaining cells, and washed a further 6-8 times in PBST to remove any remaining debris.

To develop spots, 100 μL of PBST containing either biotinylated anti-IFN-γ (XMG1.2) or biotinylated anti-IL-4 (BVD6-24G2) (Pharmingen) at a concentration of 1.25 μg/mL was added to appropriate wells of washed plates. Plates were then incubated at 4 ºC at least overnight (up to three nights), or sometimes incubated for 4 hours at room temperature. After another cycle of thorough washing in PBST (6-8 individual washes), 100 μL of alkaline-phosphatase streptavidin at a concentration of 0.2 μg/mL of PBST was added to each well and the plate(s) incubated for 1.5-2 hours at room temperature. Following this incubation, plates were thoroughly washed with ddH_2O. Finally, spots were developed via the addition to each well of 100 μL of Nitro Blue Tetrazolium Chloride and 5-Bromo-4-Chloro-3-Indolyl Phosphate (NBT/BCIP) stock solution (Boehringer Mannheim, Germany) diluted 1:50 in the development buffer (0.1 M Tris-Hcl, pH 9.5; 0.1 M NaCl; 0.05 M $MgCl_2$). After sufficient spot development (10-20 minutes), plates were washed twice in ddH_20 and allowed to dry at least overnight at room temperature. Spots were then enumerated using a dissecting microscope with the assistance of an eyepiece grid.

4.8 Spleen cell fractionation procedures

4.8.1 Depletion of spleen cell populations by antibody-dependent complement-mediated lysis

Spleen cells (prepared as described in 4.6) resuspended at $1x10^7$ cells/mL in complete RPMI media in 15 mL test tubes were treated with ascitic fluid containing either anti-CD4 (GK 1.5), anti-CD8 (Tib 211), or anti-Thy 1.2 (Tib 99) antibody. Hybridomas Tib 99 and Tib 211 were obtained from ATCC (Rockville, Maryland, USA), GK 1.5 was obtained from Dr. F. W. Fitch. The amount of ascitic fluid added was previously determined to be optimal for effective depletion. Cells and antibody were incubated on ice for 1.5 hours. Cells were then washed by flooding tubes with complete RPMI media and centrifuging for 10 minutes at 280 x g. Following this wash, cells were resuspended in rabbit complement (Cedarlane Laboratories, Hornby, Ontario, Canada) at a concentration of 1 mL of 1:20 diluted complement per $1x10^7$ cells.

Complement was prepared by resuspending lyopholized stocks in complete RPMI media, and sterilizing through a .22 μm syringe filter (Nalgene, Rochester, New York, USA). Freshly prepared complement was then incubated with a minimum of $2x10^7$ naive spleen cells/thymocytes for 30-45 minutes on ice to reduce toxicity; de-toxified complement was centrifuged for 10 minutes at 280 x g and the supernatant used as complement for subsequent depletion.

Complement was added to antibody-treated cells for one hour at 37 ºC with regular mixing every 20 minutes. Following this incubation, cells were washed twice in complete RPMI media and resuspended in fresh media. Always, control samples of cells were included which were either treated with antibody but not with complement, or were treated with complement but not with antibody. The number of viable leukocytes was assessed after depletion by trypan blue exclusion, as a rough (but reliable) indicator of percentage depletion (as compared to controls mentioned). In some cases, flow cytometry was done to determine the efficiency of depletion.

4.8.2 Spleen cell fractionation by magnetic cell sorting (MACS)

MACS microbeads (Miltenyi Biotech, Germany) were employed to isolate/remove CD4 T cells from whole spleen populations. Single cell suspensions were prepared as described (in 4.6) and RBC removed via a 3 minute incubation in Ammonium Chloride Phosphate (ACK) lysing buffer (0.15 M NH_4Cl, 1.0 mM $KHCO_3$, 0.1 mM Na_2EDTA in ddH_20; pH 7.2-7.4). Cells were washed, resuspended in MACS buffer (5% FCS in PBS), and filtered through sterile 30 μm nylon mesh. Filtered cells were washed again and resuspended in 90 μL of MACS buffer per $1x10^7$ cells.

MACS beads were added to samples as per manufacturer's instructions, mixed gently, and incubated at 6-12 ºC for 15-20 minutes. Cells were then washed, and the pellet resuspended in 1 mL of fresh MACS buffer per $1x10^8$ cells. Positively selected and negatively selected cells were then collected in separate test tubes by

harvesting effluent from MACS columns while in the presence or absence of a magnetic field (as described in procedures for the individual MACS bead kits employed).

Recovery of cells from MACS procedures varied greatly between runs employing the same bead kits, and between bead kits (55-80%); purity of positively and negatively selected cells was similarly high between all experimental runs (see Results).

4.9 Flow cytometric analysis

Flow cytometric analysis was performed on various populations to assess the effectiveness of depletion/separation procedures, and also to assess the presence or absence and/or relative size of specific sub-populations. Cells were directly stained for CD4 (clone RM4-4, recognizing a different epitope than clone GK 1.5), CD8, Thy 1.2, B220, CD19, and the DO11.10 clonotypic TCR (KJ1-26) with monoclonal FITC or PE conjugated antibodies (Cedarlane Laboratories). Generally, after thorough washing, $1x10^5$ -$1x10^6$ cells in 100 μL of FACS buffer (2% FCS in PBS) were incubated with 2-10 μL of antibody for 30 minutes in darkness, on ice. When double-staining was done on cells, antibodies were added one at a time, as described above, with a wash step in between. Stained cells were washed twice in 1 mL of fluorescence-activated-cell-sorting (FACS) buffer and were resuspended in either 0.5-1.5 mL of FACS buffer directly (when analyzed the same day), or were resuspended in 100 μL of FACS buffer and an equal volume of FACS fixative

solution - PBS and 2% formaldehyde (when analyzed the following day; in this case, the final volume was brought to 0.5-1.5 mL with FACS buffer before analysis). Before analysis, samples were often filtered through 30 μm nylon mesh to remove any small aggregates, judged capable of potentially 'clogging' the Flow Cytometer. Analysis was done using a Beckman Coulter Epics Flowcytometer and with Expo32 v. 1.2 analysis software (Beckman Coulter, Mississauga, Ontario, Canada).

5.0 Results

5.1 Layout of the report of experimental results

I chose to divide the Results section of this thesis into three major sub-sections. I will begin by describing and explaining in brief the reasoning behind the choice of experimental system, the antigens employed, and the readout assay used to analyze immune responses (5.2), thus validating this approach as one capable of examining variables affecting the Th1/Th2 phenotype of a primary response (5.3). I will then present experiments and data attempting to dissect some of the most important aspects of the mechanisms by which these variables of immunization, introduced in section 5.3, influence the Th1/Th2 phenotype of the response (5.4). Finally, I will describe experiments in which I have attempted to better characterize the cellular interactions involved and found to be critically important in section 5.4, in part by refining populations used to reconstitute lethally irradiated mice challenged with antigen (5.5).

5.2 Choice of experimental system, antigens, and readout assay

In order to test various aspects of the Th1/Th2 decision criterion, it is necessary to employ an experimental system that allows the manipulation of discrete, single variables in combination with a non-immunomodulatory antigen. An adoptive transfer system, in which naive mice are lethally irradiated and then reconstituted with different numbers and types of cells from naive donors, offers a sensitive *in vivo* setting in which the variables of antigen dose, and of the number of responding CD4 T cells (and potentially other cellular subsets as well), can be manipulated independently (Bretscher, 1983; Ismail and Bretscher, 2001). Importantly, lethal irradiation does not seem to create an 'empty' environment favorable to a high degree of lymphopenia-induced or homeostatic proliferation of T cells, as compared to other, similar environments, such as RAG-deficient or TCR-deficient animals (Kieper et al., 2005); a strong lymphopenia induced proliferation by transferred T cells could impact the responses observed after 7 days via variables other than those controlled for. To determine the Th1/Th2 phenotype of responses generated, an optimized ELISPOT assay is employed – the number of antigen-dependent IFN-γ and IL-4 spots observed in the assay is taken as a measure of the Th1 and Th2 elements of the response (Power et al., 1999). Importantly, in this optimized ELISPOT assay, the number of antigen-dependent spots is directly proportional to the number of immune cells plated (Power et al., 1999).

The use of XRBC as antigen is particularly suitable in this system as XRBC are immunogenic without adjuvant and have no overt immunomodulatory effects, especially with regards to the Th1/Th2 phenotype of a concurrent, non-crossreacting immune response (Lagrange et al., 1974; Ismail and Bretscher, 1999). The use of adjuvants, even if not overtly pro Th1/Th2 in nature, might well impact the immune system's Th1/Th2 phenotypic choice in a manner outside of the influence of variables we wished to address, namely, the effects of the dose of antigen and the number of responding CD4 T cells. Numerous observations suggest that both cell-mediated and humoral immunity can be generated against XRBC, and that this Th1/Th2 phenotype is largely antigen dose-dependent, with higher doses favoring Th2 responses (see 1.10.5). Importantly, coupling a smaller protein, such as HEL or OVA, to XRBC populations does not alter the dose dependence of the XRBC-specific Th1/Th2 response when used to challenge intact mice (Ismail, 2000; Ismail et al., 2005).

The number of raw antigen-dependent IFN-γ and IL-4 producing cells observed in the ELISPOT assay is determined by subtracting spots generated in the absence of antigen (background) from the number of spots generated in wells containing relevant antigen. At least three wells per condition (antigen + and -) were counted and averaged in all experiments. Importantly, unimmunized but reconstituted control mice were included in all experiments to rule out cytokine production in response to antigen in the ELISPOT assay, due to unforeseen events after reconstitution, such as activation of transferred cells specific for a cross-

reactive antigen, for example, a damaged-self determinant. These control mice consistently generated negligible numbers of antigen-dependent spots.

The number of raw antigen-dependent spots observed in the ELISPOT assay has been used to calculate the number of antigen-specific, cytokine-producing cells produced per million unprimed responding cells employed for reconstitution of lethally irradiated mice. This parameter gives a measure of the relative efficiency with which the CD4 T cells, given to reconstitute irradiated mice, are activated to produce cytokine-producing cells under the different circumstances examined. This 'normalized' response per million cells input reflects this calculation and is presented in most figures. The recovery of spleen cells at 6 or 7 days post-immunization was generally 75 -150% of input in mice reconstituted with $2\text{-}3x10^7$ naive spleen cells.

As discussed in 5.3.2, to determine if the number of antigen-specific CD4 T cells present in the reconstituting population of naive cells given to irradiated, challenged mice was a critical variable affecting the Th1/Th2 phenotype of the response generated, we sometimes employed small numbers of spleen or thymus cells from a mouse transgenic for an MHC II restricted TCR. No such mouse with a TCR specificity for XRBC has been created. We therefore employed the DO11.10 mouse, on a BALB/c background ($H\text{-}2^d$) with a transgenic TCR recognizing OVA (Murphy et al., 1990). By immunizing with the conjugate antigen XRBC-OVA, which the immune system sees as one singular nominal antigen, OVA-specific CD4 T cells can be viewed as specific for this 'neo' antigenic whole. This approach has proven successful in a similar but slightly different setting, and additionally, it offers

the opportunity to study defined cooperative events in the generation of immune responses (Ismail, 2000; Ismail et al., 2005).

It is a common finding that commercially available proteins, such as OVA and HEL, are often contaminated with variable amounts of LPS (Watanabe et al., 2003; Reis e Sousa and Germain, 1999). In different experimental systems, LPS contamination of protein antigen has been found to cause significant system-wide (Pulendran et al., 2001; Watanabe et al., 2003), or targeted (Reis e Sousa and Germain, 1999) effects, or has been found to cause no significant effect (Peters, 2002). These observations suggest that coupling LPS-containing OVA to SRBC could result in an antigen with inherent immunomodulatory properties, especially as different microbial sources of LPS have been found to induce different cytokine profiles when used as adjuvants (Pulendran et al., 2001). Importantly, in preliminary experiments conducted employing intact BALB/c mice injected with either uncoupled or OVA-coupled SRBC, the predominant Th1/Th2 phenotype of the SRBC-specific response was similar when the same dose of SRBC or SRBC-OVA was used (not shown). Furthermore, the Th1/Th2 phenotype of the SRBC-specific response was dependent on the immunizing dose (lower doses correlating with IFN-γ production and higher doses with IL-4 production) (not shown). These and other preliminary experiments, undertaken while optimizing the coupling protocol, suggest that LPS contamination of OVA does not *overtly* influence the Th1/Th2 phenotype of antigen-dependent cytokines produced in this experimental system.

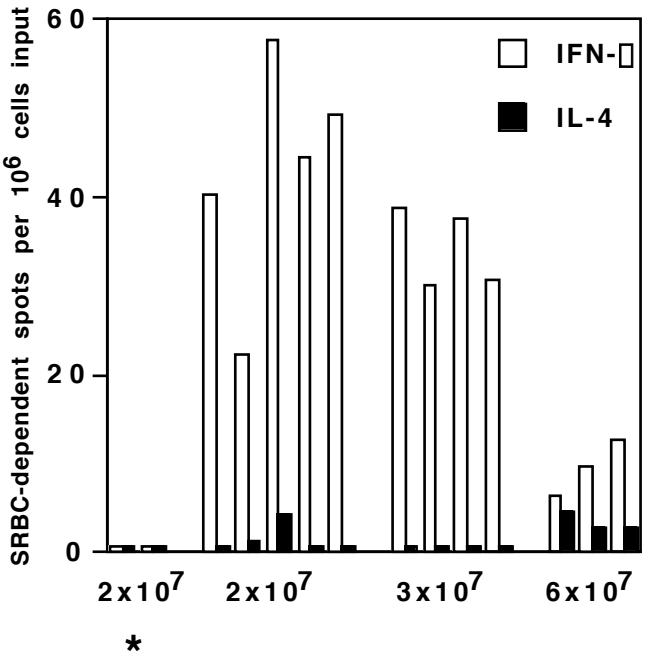
5.3 Variables influencing the Th1/Th2 phenotype of an antigen-specific response in the adoptive transfer system

5.3.1 Defining a predominantly Th1, primary XRBC-specific response in the adoptive transfer system

To investigate the Th1/Th2 decision criterion, it was necessary to determine a dose of coupled antigen (XRBC-OVA), and a reconstituting number of normal, naive spleen cells which would result in the generation of a predominantly Th1 antigen-specific response, characterized by the presence of antigen-dependent IFN-γ, and the (relative) absence of antigen-dependent IL-4 spots. When challenged with an arbitrarily 'high' dose of antigen, 200 μL of .1% SRBC-OVA, lethally irradiated mice reconstituted with either 2 or $3x10^7$ normal spleen cells generate a predominant Th1 response, characterized by significant numbers of antigen-dependent IFN-γ and few antigen-dependent IL-4 spots, as assessed six days after immunization (Figure 1). Similar experiments were done on day 5 and 7 post-immunization and also resulted in Th1 anti-SRBC responses, demonstrating a predominant Th1 response phenotype throughout the peak period of antigen-specific responses in this experimental system (see next sub-section). Interestingly, when mice were reconstituted with $6x10^7$ normal spleen cells, the ratio of IFN-γ to IL-4 antigen-dependent spots is significantly lower, due to an increased number of IL-4 spots and a dramatic reduction of IFN-γ spots (Figure 1); the *normalized* response in

Figure 1: SRBC-specific responses obtained from mice immunized with a standard dose of SRBC-OVA but reconstituted with different numbers of unprimed normal spleen cells.

Lethally irradiated naive mice were reconstituted with either $2x10^7$, $3x10^7$, or $6x10^7$ naive syngeneic spleen cells (as indicated). Except for control mice reconstituted with $3x10^7$ spleen cells, but which were not immunized (*), mice were immunized with 200 μL of a .1% SRBC-OVA suspension at the time of reconstitution. 7 days after immunization, spleen cells were removed, counted, and employed in an ELISPOT assay. The number of IFN-γ (white) and IL-4 (black) SRBC-dependent spots per million cells input is presented for each individual mouse. This data represents an individual example of three similar experiments.



note: ' * ' symbol represent reconstituted, unimmunized controls

these animals, both IFN-γ and IL-4 production, is quite low, due to a significantly lower recovery of cells from the spleens of these animals at day 6. This finding could reflect the relatively low number of SRBC-specific CD4 T cells present in the spleen of naive BALB/c mice, and the resulting relatively poor efficiency of CD4 T cell activation in mice reconstituted with $6x10^7$ normal spleen cells and challenged with this particular dose of antigen.

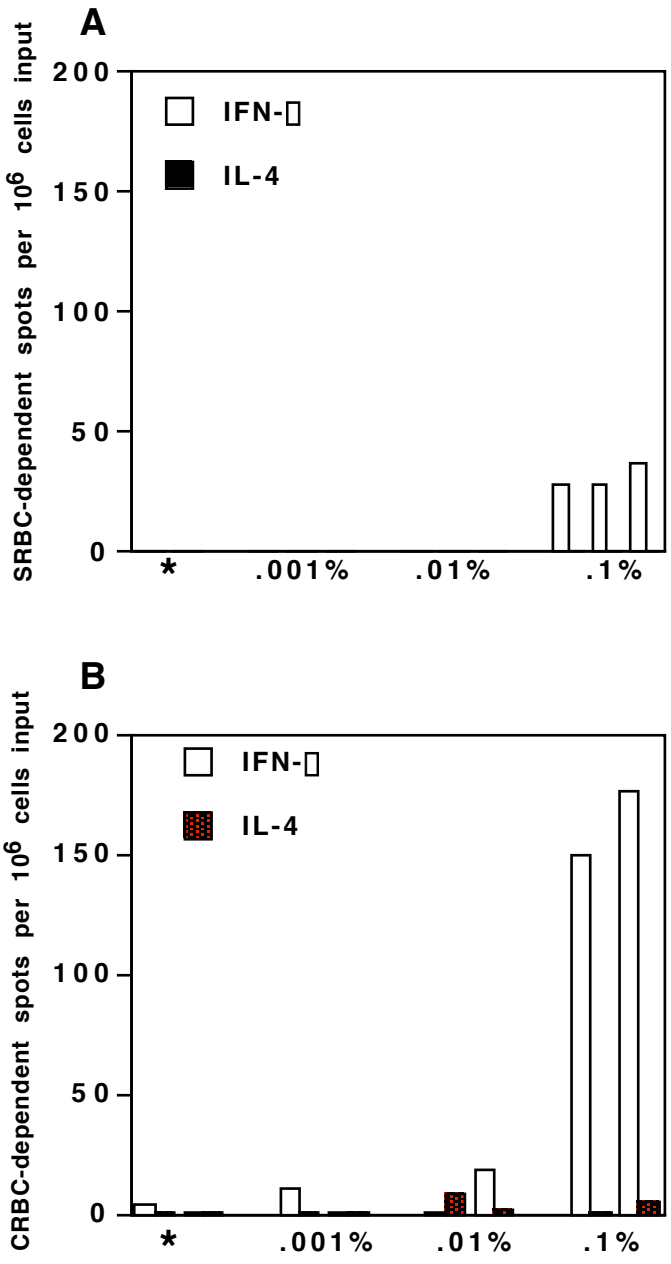
While mice reconstituted with $2x10^7$ normal spleen cells generate a predominant Th1 response when challenged with 200 μL of .1% SRBC-OVA, challenge with the same volume of .01% or .001% SRBC-OVA (Figure 2a) or CRBC-OVA (Figure 2b) does not result in a consistent, reliably detectable immune response. By manipulating the number of spleen cells used to reconstitute lethally irradiated mice, and by varying the dose of antigen used to challenge such reconstituted mice, a predominant anti-XRBC Th1 response, or a situation in which no consistently detectable anti-XRBC response is generated, can be defined.

5.3.2 The DO11.10 mouse

To investigate whether increasing numbers of antigen-specific CD4 T cells, and the resulting increases in antigen-mediated CD4 T cell – CD4 T cell interactions, are able to influence the Th1/Th2 phenotype of the XRBC-specific immune response, DO11.10 spleen cells were used to 'spike' populations of normal spleen cells given to lethally irradiated mice challenged with XRBC-OVA. As described in Materials and Methods, DO11.10 mice, on a BALB/c background ($H\text{-}2^d$),

Figure 2: Lethally irradiated mice reconstituted with $2x10^7$ naive spleen cells generate an XRBC-specific immune response when challenged with 200 μL of .1% XRBC-OVA

Lethally irradiated naive mice were reconstituted with $2x10^7$ naive spleen cells and were challenged with 200 μL of either .001%, .01%, or .1% SRBC-OVA (**A**) or CRBC-OVA (**B**). Control mice were reconstituted, but not challenged with antigen (*). 6 days after immunization, spleen cells were removed, counted, and employed in an ELISPOT assay. The number of IFN-γ (white) and IL-4 (black) SRBC-dependent spots per million cells input is presented for each individual mouse. This data represents an individual example six similar experiments. CRBC-dependent responses were consistently greater than SRBC-dependent responses. Though the *n* is only 2 in (**B**), a similar dose-dependent anti-CRBC response as depicted was noted in several other experiments, which are not shown.



note: '□' symbols represent reconstituted, unimmunized controls

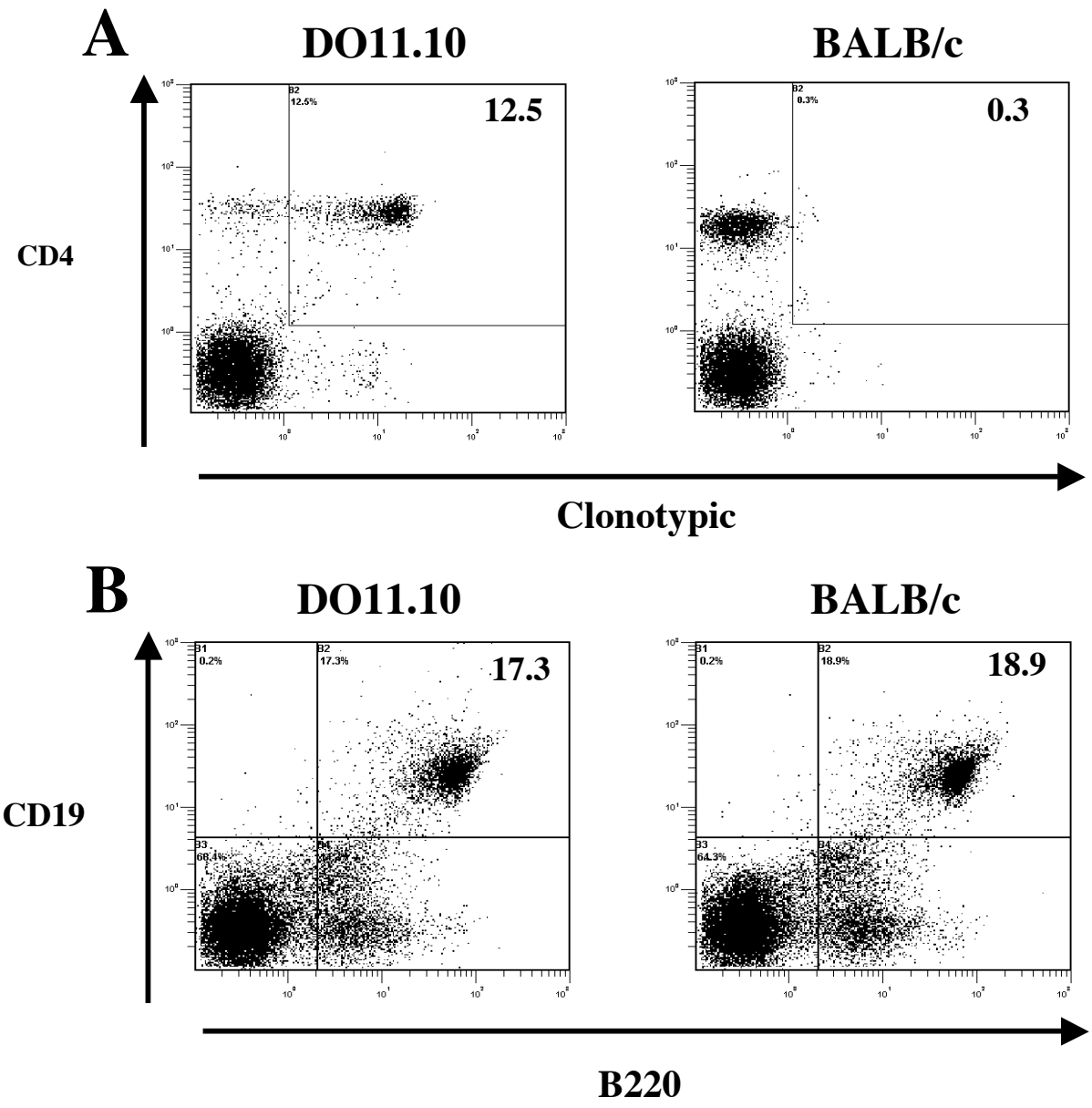
express a transgenic □□ TCR recognizing an immunodominant OVA peptide (aa 323-339) in the context of MHC II (Murphy et al., 1990; Robertson et al., 2000). I reasoned that a relatively small number of antigen-specific CD4 T cells might influence the Th1/Th2 phenotype of the anti-XRBC-OVA response with greater efficiency than that seen when a much larger number of normal BALB/c spleen cells is used for reconstitution (as in Figure 1).

Generally, naive DO11.10 mice contain 12-20% transgene expressing CD4 T cells in the spleen, as determined by FACS analysis (Figure 3a). Because B cells might play a prominent role not only in T cell activation, but also in Th1/Th2 development (see discussion), and because it has been recently reported that some T cell transgenic strains have abnormal numbers of B cells compared to wild-type mice of the same genetic background (Lobito et al., 2004), the number of CD19/B220 double-positive spleen cells, representing mostly B cells, was compared in naive BALB/c and DO11.10 mice. No difference in this population of spleen cells was observed between these two strains (Figure 3b).

Because our colony of is not derived from founder DO11.10 mice on a RAG^- background, and it is therefore possible that some CD4 T cells could express second TCRs with specificities for other antigens (including XRBC), lethally irradiated BALB/c mice were reconstituted with $2x10^7$ naive DO11.10 spleen cells and challenged with various concentrations of SRBC. Mice reconstituted with only DO11.10 cells do not generate significant SRBC-specific cytokines (results not shown). Furthermore, DO11.10 spleen cells, while responding vigorously *in vitro* when challenged with soluble OVA or SRBC-OVA, did not generate significant

Figure 3: A comparison between splenic CD4/clonotypic TCR double-positive and CD19/B220 double-positive populations in age-matched DO11.10 and BALB/c mice

Age matched, naive, female DO11.10 and BALB/c mice were sacrificed, their spleens removed, and samples prepared for FACS analysis, using relevant PE- (CD4 or CD19) and FITC- (Clonotypic, B220) conjugated antibodies, as described (controls not shown). The percentage of transgenic TCR bearing CD4+ T cells is given for both DO11.10 and BALB/c mice (**A**) as is the percentage of CD19+, B220+ double-staining spleen cells (**B**).


A
DO11.10
BALB/c
12.5
0.3
CD4
Clonotypic
B
DO11.10
BALB/c
17.3
18.9
CD19
B220

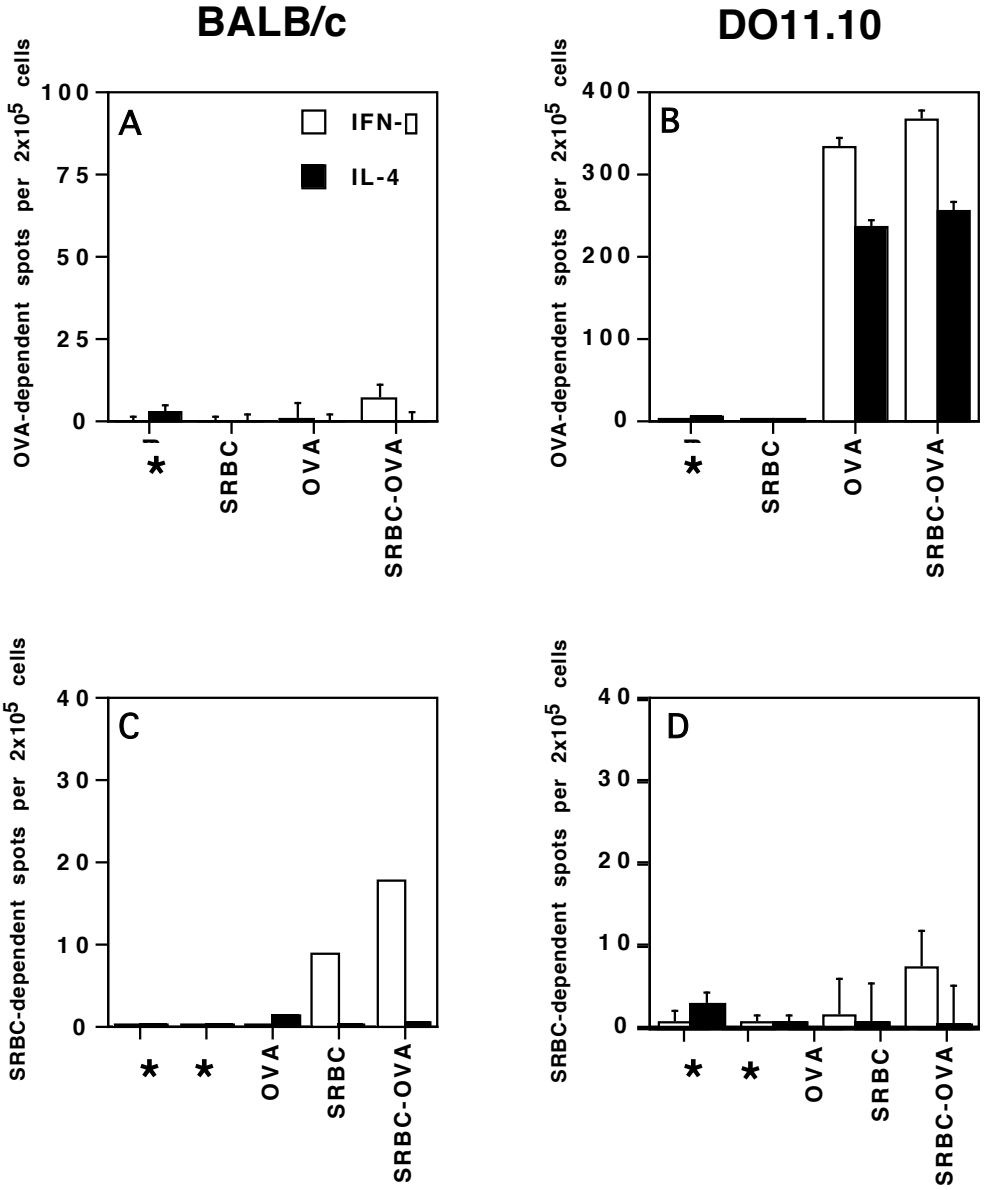
numbers of SRBC-specific spots when challenged with either SRBC or SRBC-OVA (Figure 4). Based on these findings, any effect on the XRBC-specific Th1/Th2 phenotype generated against XRBC-OVA when lethally irradiated mice are reconstituted with, in addition to a set number of normal spleen cells, a small number of DO11.10 spleen cells, would seem to reflect an impact of DO11.10, OVA-specific spleen cells, on the activation of T cells present in the normal spleen specific for XRBC determinants. Several aspects of the cooperation between these two populations affecting the Th1/Th2 phenotype of the XRBC-OVA immune response are presented in the following sections.

5.3.3 A small number of DO11.10 spleen cells can modulate a predominant Th1 XRBC-specific immune response to a mixed Th1/Th2 phenotype

I wished to test the prediction that more antigen-mediated CD4+ T cell interactions are needed to generate Th2 responses than Th1 responses. I therefore reconstituted irradiated mice with either 2 x 10^7 syngeneic spleen cells alone, or with these cells and a small number, 1 x 10^6, of unprimed spleen cells from a DO11.10 donor. Such mice, receiving a 5% 'spike' of transgenic cells in addition to 2x10^7 normal cells, contain a small but detectable number of transgenic CD4 T cells in the reconstituting population of spleen cells (Figure 5). Importantly, I chose to employ a limiting number of transgenic spleen cells that reproducibly affect the SRBC-specific response upon immunization with SRBC-OVA. I wished to avoid the use

Figure 4: Characterization of *in vitro* SRBC-, OVA-, and SRBC-OVA-specific IFN-γ and IL-4 responses generated by BALB/c or DO11.10 spleen cell cultures

$3x10^6$ naive BALB/c (**A** and **C**) or DO11.10 (**B** and **D**) spleen cells per well were cultured for 5 days in 24-well plates in the presence of either no antigen (*), SRBC, OVA, or SRBC-OVA. IFN-γ (white) and IL-4 (black) OVA-dependent (**A** and **B**) and SRBC-dependent (**C** and **D**) responses are presented per $2x10^5$ cultured cells recovered on day 5. Note differences in scale between panels. This experiment is representative of several (over ten) individual experiments. Error bars represent +/- SD of values from triplicate wells plated in the ELISPOT assay.

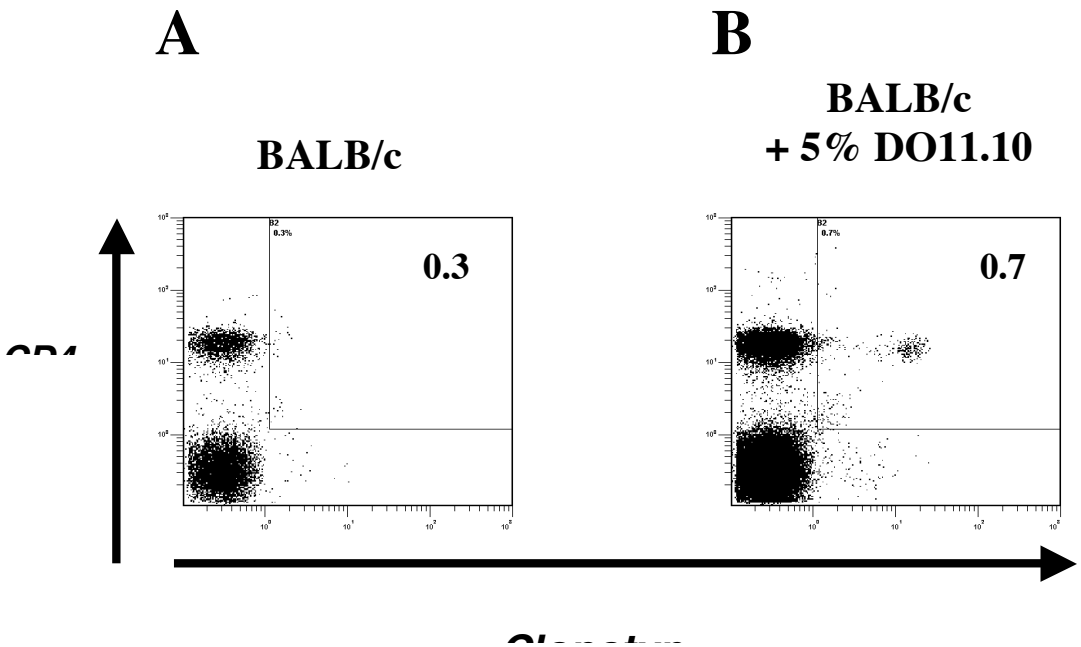


Antigen in Culture

note: '□' symbols represent reconstituted, unimmunized controls

Figure 5: Profiles of representative reconstituting populations of spleen cells employed in adoptive transfer experiments as determined by flow cytometry (Day 0)

Prior to injection into lethally irradiated hosts, samples of representative reconstituting spleen cell populations containing either only $2x10^7$ normal naive spleen cells (**A)** or containing $2x10^7$ normal spleen cells and an additional population of five percent ($1x10^6$) naive DO11.10 spleen cells (**B**) were removed and stained with PE-conjugated anti-CD4 and FITC-conjugated anti-clonotypic TCR antibodies and analyzed by FACS. Controls are not shown.


A
B
BALB/c
BALB/c
+ 5% DO11.10
0.3
0.7

of more transgenic OVA-specific CD4 T cells, a situation where the anti-OVA response might overwhelm the response against SRBC. Results of experiments tracking the number of OVA-specific (clonotypic TCR-positive) CD4 T cells show that reconstituting mice with normal BALB/c spleen cells and $1x10^6$ transgenic spleen cells results in a barely detectable population on day 7 after immunization, as assessed by FACS analysis (not shown).

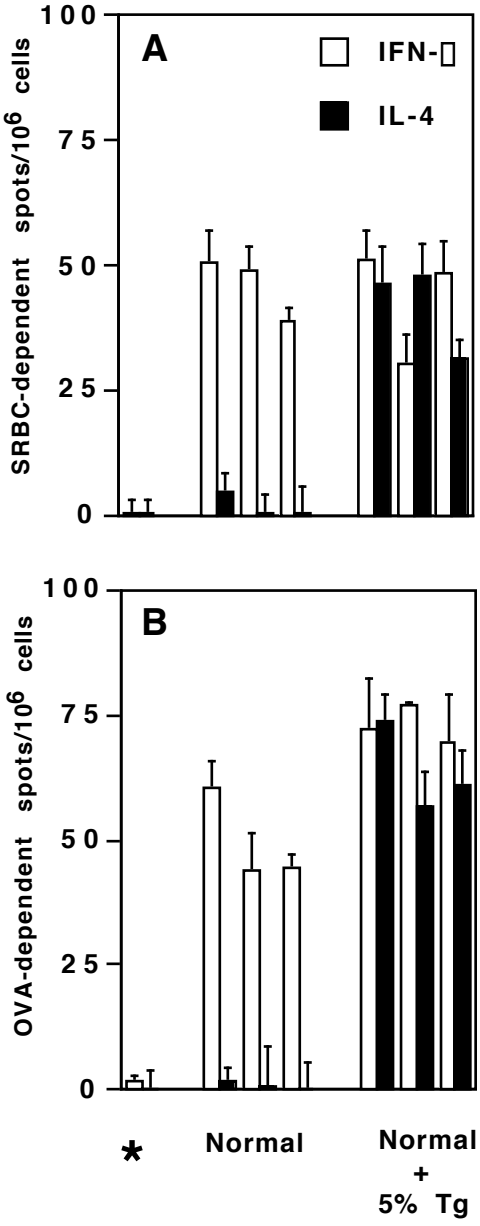
If the addition of this relatively small number of DO11.10 spleen cells is able to modulate the overall Th1/Th2 phenotype of the SRBC-specific CD4 T cell response, the majority of which is presumably mediated by cells in the reconstituting normal spleen population, then it most likely represents cooperative events between these populations, leading to differential activation of SRBC-specific cells, as the presence of an additional $1x10^6$ spleen cells in general is unlikely to influence the response as shown in Figure 1.

The response of mice reconstituted with only normal spleen cells and challenged with 200 μL of .1% SRBC-OVA consisted predominantly of antigen-specific Th1 cells, as expected, whereas those reconstituted with additional OVA-specific transgenic cells contained significant numbers of SRBC- and OVA-specific IL-4 producing cells (Figure 6 a and b). In experiments aimed at defining a minimum number of DO11.10 spleen cells able to modulate the SRBC-specific Th1/Th2 phenotype, reconstitution with fewer than $1x10^6$ (5%) DO11.10 spleen cells did not reliably affect the SRBC-specific Th1/Th2 phenotype (not shown).

The predominant Th1/Th2 phenotype of an immune response can change dramatically through the course of the response, as many humoral (i.e., Th2)

Figure 6: **The presence of a small number of DO11.10 spleen cells causes a SRBC-OVA-specific, predominant Th1 response to switch towards a mixed Th1/Th2 phenotype**

Lethally irradiated mice were reconstituted with either $2x10^7$ naive spleen cells alone, or with $2x10^7$ naive spleen cells and $1x10^6$ DO11.10 spleen cells. Except for control mice, which were not immunized (*), mice recieved 200 μL of a .1% SRBC-OVA solution at the time of reconstitution. 6 days after immunization, spleen cells were removed, counted, and employed in an ELISPOT assay. The number of IFN-γ (white) and IL-4 (black) SRBC-dependent spots (**A**) and OVA-dependent spots (**B**) per million cells input is presented for each individual mouse. This data represents one of over ten individual experiments.



note: '□' symbols represent reconstituted, unimmunized controls

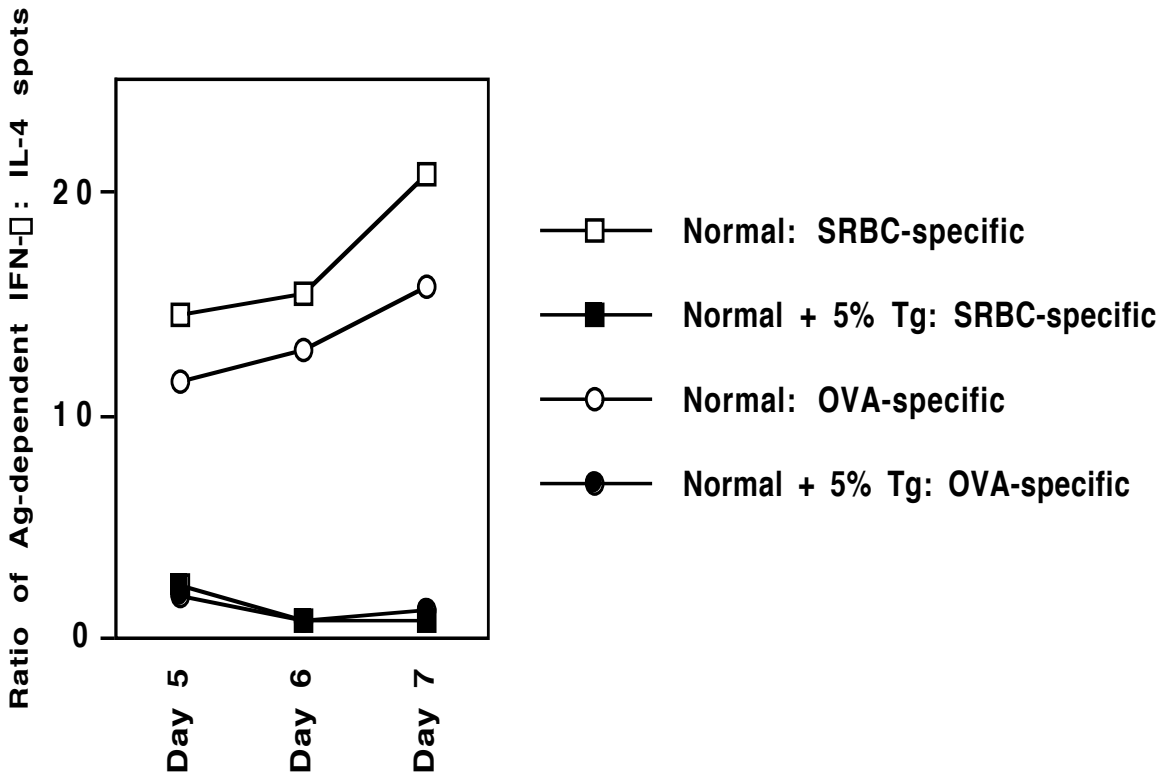
responses are preceded by a period characterized by cell-mediated immunity (Salvin, 1958). To rule out kinetic changes in the SRBC-OVA Th1/Th2 response phenotype, the ratio of IFN-γ to IL-4 spots specific for SRBC and OVA was determined throughout the maximal response period. As seen in figure 7, mice receiving additional DO11.10 spleen cells generated higher numbers of IL-4 producing cells, resulting in a lower IFN-γ: IL-4 ratio on days 5, 6, and 7 post immunization.

5.3.3.1 Coherence between the XRBC-specific and OVA-specific Th1/Th2 phenotype generated upon challenge with XRBC-OVA

It has been suggested that cooperation between CD4 T cells specific for the same nominal antigen, as demonstrated in Figures 6 and 7, can explain why the Th1/Th2 phenotype of CD4 T cells specific for peptides derived from the nominal antigen tend to be similar under a given set of circumstances (Bretscher et al., 2001). This coordinated regulation of the Th1/Th2 phenotype of T cells specific for diverse peptides of the nominal antigen has been referred to as 'coherence' (Bretscher, 1989). The observed coherence between the Th1/Th2 phenotype of the anti-XRBC response and the anti-OVA response was a consistent finding in all experiments employing the conjugate antigen, similar to the coherence seen in responses to other 'linked' antigens (Gerloni et al., 2000), and to other, more complex antigens, such as mycobacteria and leishmania parasites (Bretscher et al., 2001). In the following

Figure 7: The antigen-specific Th1/Th2 response phenotype is stable over the peak response period

The average ratio (three mice per group) of IFN-γ to IL-4 spots observed in the ELISPOT using SRBC (squares) or OVA (circles) as antigen is shown for days 5-7 post-immunization from lethally irradiated mice reconstituted with only normal spleen cells (open) and with an additional $1x10^6$ DO11.10 spleen cells (filled). All mice were challenged with 200 μL of a .1% SRBC-OVA solution at the time of reconstitution. Unimmunized controls are not shown, but did not produce significant antigen-dependent spots. As indicated in the note under Figure 7, no responses produced a ratio of IFN-γ to IL-4 spots less than 0.7, indicating that relatively pure IL-4 responses were not observed at any time.



Note: No IFN-γ:IL-4 ratio values are below .70

experiments, we report primarily on the phenotype of the XRBC-specific responses generated, as we concentrated on the mechanism whereby OVA-specific DO11.10 spleen cells influenced the XRBC-specific response when irradiated, reconstituted mice were immunized with XRBC-OVA.

5.3.4 A small number of DO11.10 spleen cells can facilitate the generation of a predominant Th1 XRBC-specific response when adoptively transferred mice are challenged with a normally sub-immunogenic dose of XRBC-OVA

The effect of the additional DO11.10 spleen cells, in supporting the generation of anti-SRBC Th2 cells, could be due to more effective antigen-specific CD4 T cell cooperation, or it could reflect the existence of cells in the transgenic spleen with an inherently Th2 promoting activity, such as OVA-specific CD4 T cells producing IL-4 (Gollob and Coffman, 1994; Creusot et al., 2003), or Th2 polarizing APC (MacDonald and Pearce, 2002). In this series of experiments, I tested a prediction unique to the hypothesis that more CD4 T cell – CD4 T cell interactions are required to generate Th2 than Th1 cells. Lethally irradiated mice were reconstituted, as before, with or without an additional $1x10^6$ DO11.10 spleen cells, but were immunized with 200 □L of .01% SRBC-OVA, a dose found in preliminary experiments to be too low to consistently generate detectable immune responses in mice reconstituted with only 2 or $3x10^7$ normal spleen cells. Mice reconstituted with both normal spleen cells and $1x10^6$ additional DO11.10 spleen cells reliably

produced SRBC-specific Th1 responses, as characterized by significant numbers of IFN-γ producing cells, and few, if any, antigen-specific cells producing IL-4 (Figure 8). These observations demonstrate that the same number of transgenic cells can aid in the generation of either Th1 or Th2 anti-SRBC cells, depending on the dose of immunizing antigen. This finding is not explicable on the hypothesis that $1x10^6$ transgenic spleen cells contain cells uniquely promoting either Th1 or Th2 responses. They further demonstrate that both the generation of Th1 and Th2 cells requires CD4 T cell cooperation.

5.3.5 The antigen dose and number of antigen-specific CD4 T cells present at the time of immunization influence the Th1/Th2 phenotype co-jointly

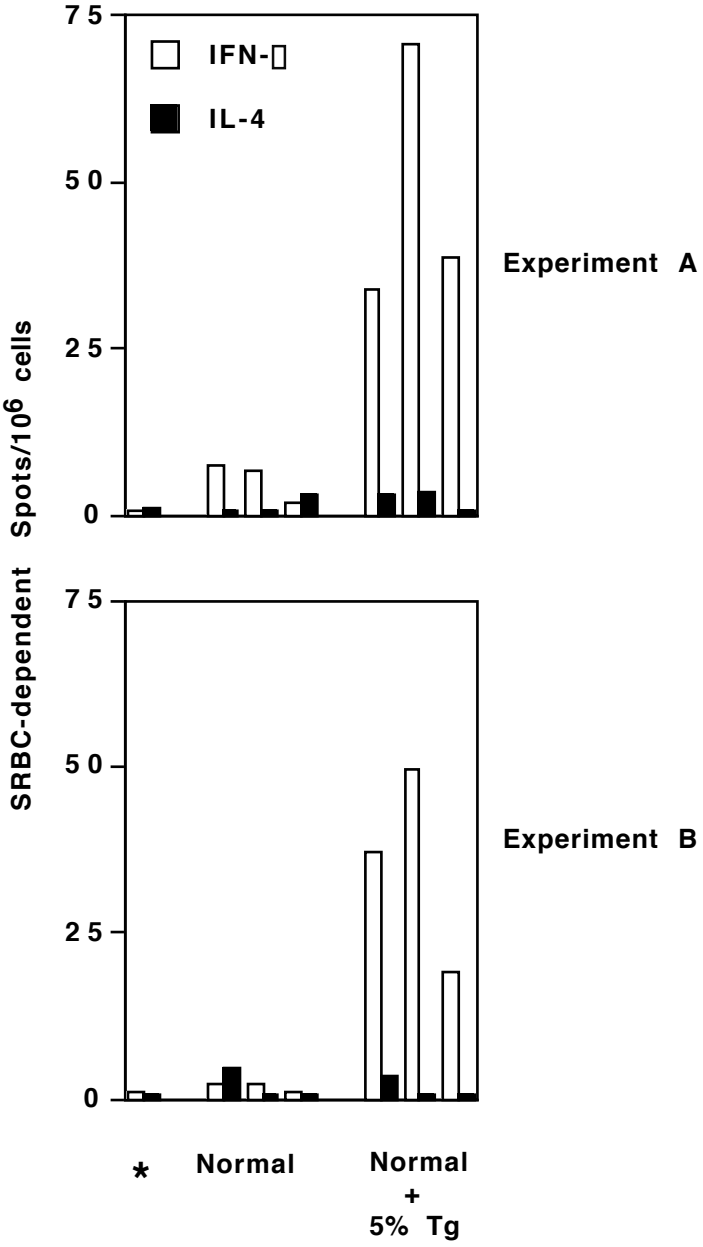
The results summarized to this point support predictions made by the Threshold Hypothesis (see 2.0). In the presence of a given number of antigen-specific CD4 T cells, an arbitrarily 'high' dose of XRBC-OVA can generate a predominant Th1 response. By increasing the number of antigen-specific CD4 T cells able to cooperate in the initiation of the antigen-specific response, a significant number of Th2 cells are generated. However, if the amount of antigen is decreased, limiting overall antigen-mediated CD4 T cell cooperative events and thus not facilitating the generation of a detectable immune response, then, in the presence of increased number of antigen-specific CD4 T cells, a Th1 response can be generated due to significant, but not optimal cooperative events. This interdependent

relationship between antigen dose and the number of responding T cells is evident from the observations presented in Figure 9, depicting two of several representative experiments employing either SRBC-OVA and CRBC-OVA as antigen.

A note on the differential length (6 or 7 days) of adoptive transfer experiments depicted. During the course of my initial experiments, I noted that in mice which were immunized and reconstituted identically, the number of antigen-dependent IL-4 producing cells observed in the ELISPOT assay was often slightly higher on day 7 than on day 6, while the number of IFN-γ spots was similar on both days. For this reason, most experiments in which IL-4 was assayed were conducted 7 days after reconstitution and immunization, so as to optimize IL-4 detection (while not appreciably sacrificing IFN-γ detection). Preliminary experiments conducted 8 days after reconstitution and immunization often yielded very low antigen-specific responses (results not shown). Similarly, experiments conducted 5 days after reconstitution and irradiation yielded significantly lower responses (but of the same general Th1/Th2 phenotype, see Figure 7) than experiments conducted on day 6 or 7.

Figure 8: The presence of a small number of DO11.10 spleen cells, in addition to $2x10^7$ normal spleen cells, results in the generation of a SRBC-specific Th1 response when mice are immunized with a normally sub-immunogenic dose of SRBC-OVA

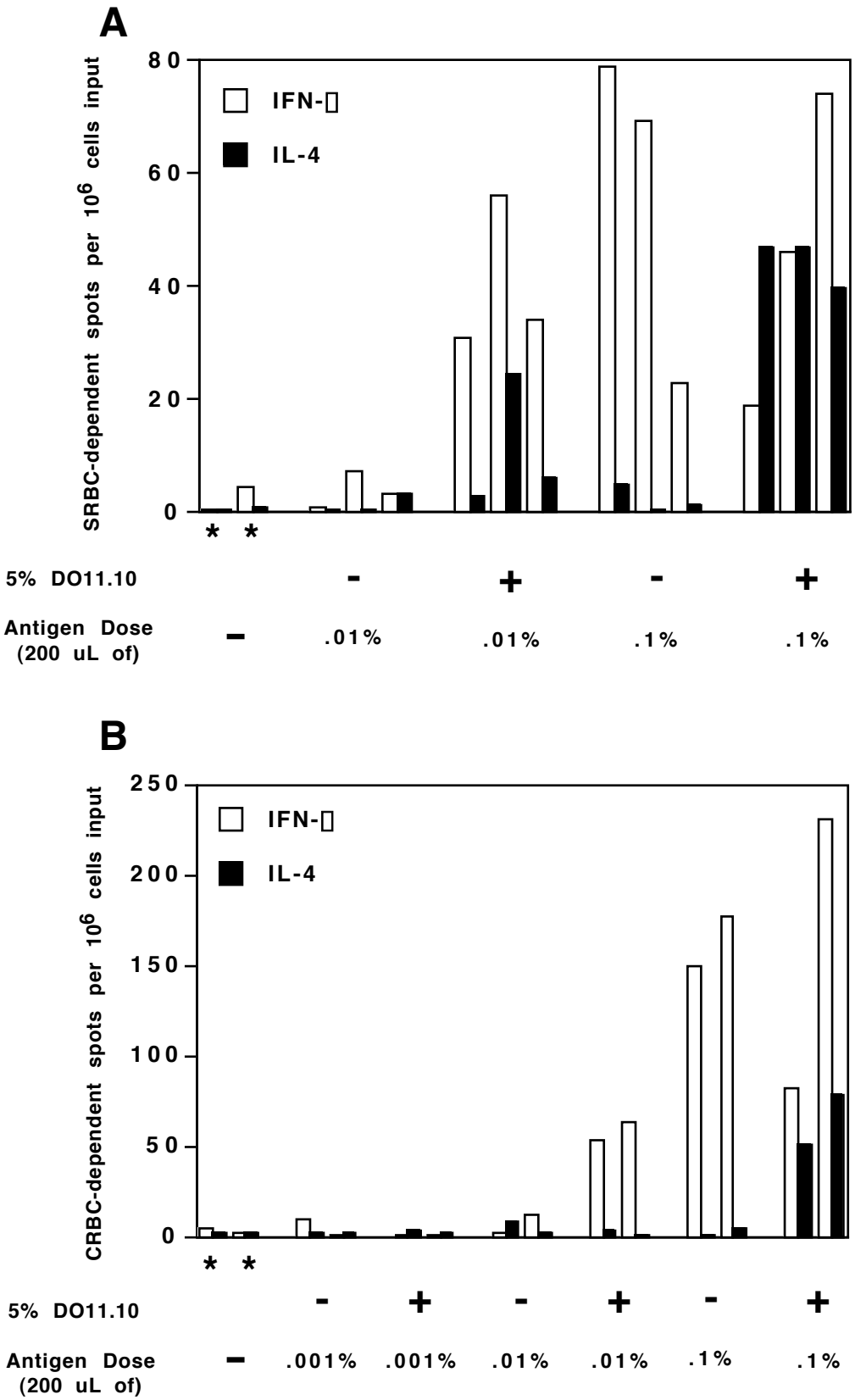
Lethally irradiated mice were reconstituted with either $2x10^7$ naive spleen cells alone, or with $2x10^7$ naive spleen cells and $1x10^6$ naive DO11.10 spleen cells. Except for control mice, which were not immunized (*), mice received 200 μL of a .01% SRBC-OVA solution at the time of reconstitution. 6 days after immunization, spleen cells were removed, counted, and employed in an ELISPOT assay. The number of IFN-γ (white) and IL-4 (black) SRBC-dependent spots per million cells input is presented for each individual mouse. Data from two separate experiments is presented.



note: '□' symbols represent reconstituted, unimmunized controls

Figure 9: The Th1/Th2 phenotype of the XRBC-specific immune response is co-dependent on the number of antigen-specific CD4 T cells and the antigen dose

Lethally irradiated mice were reconstituted with either $2x10^7$ naive spleen cells alone, or with $2x10^7$ naive spleen cells and $1x10^6$ DO11.10 spleen cells (as indicated). Except for control mice, which were not immunized (*), mice received 200 μL of various concentration of XRBC-OVA at the time of reconstitution (as indicated). 7 days after immunization, spleen cells were removed, counted, and employed in an ELISPOT assay. The number of IFN-γ (white) and IL-4 (black) SRBC-dependent spots (**A**) and CRBC-dependent spots (**B**) per million cells input is presented for each individual mouse. Note the differences in scale, reflecting the difference in magnitude between the SRBC- and CRBC-specific response (as introduced in figure 2).

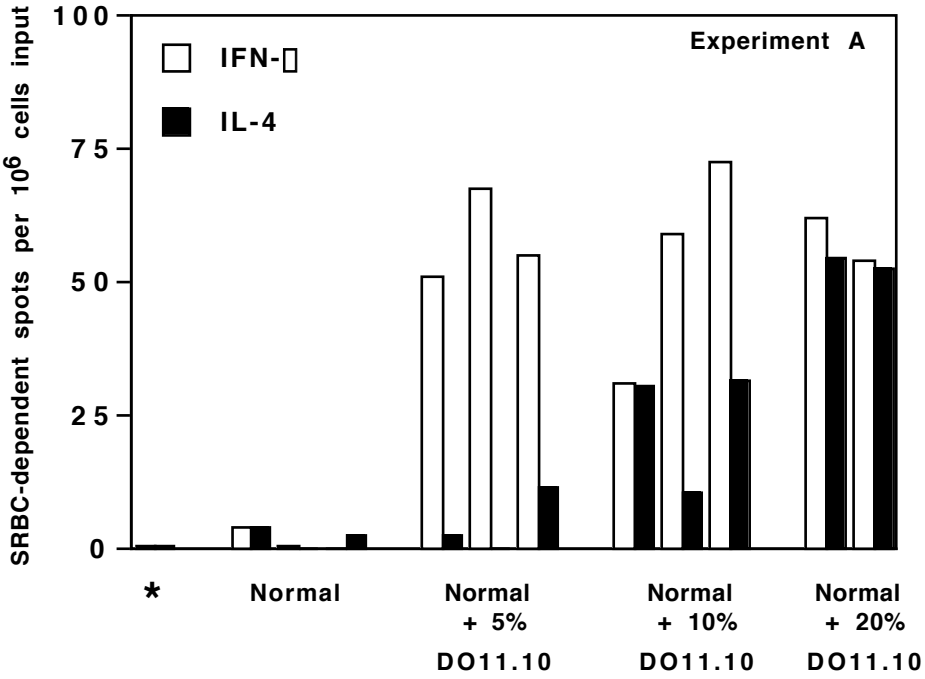

A
SRBC-dependent spots per 10^6 cells input
80
60
40
20
0
IFN-γ
IL-4
* *
5% DO11.10
- + - +
Antigen Dose (200 uL of)
- .01% .01% .1% .1%
B
CRBC-dependent spots per 10^6 cells input
250
200
150
100
50
0
IFN-γ
IL-4
* *
5% DO11.10
- + - + - +
Antigen Dose (200 uL of)
- .001% .001% .01% .01% .1% .1%

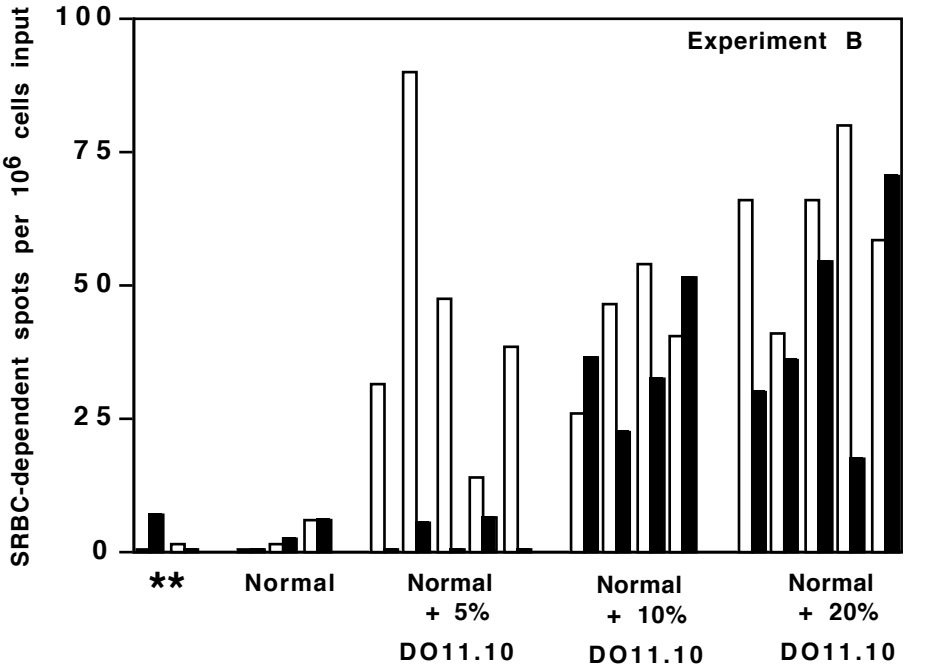
5.3.6 Increasing numbers of DO11.10 spleen cells, used to 'spike' the reconstituting population of normal spleen cells, progressively support the generation of Th1 and Th2 XRBC-specific cells in the adoptive transfer system

The results presented in Figure 9 could potentially be explained by the differential activation, depending on antigen dose, of the same or of different populations of cells in the transgenic spleen cell population towards a Th1 or Th2 promoting phenotype. This could be because of some adjuvant-like quality of XRBC, which, when present above a certain threshold, as in the 'high' dose versus the 'low' dose of conjugate, would promote Th2 responses, regardless of other variables. To rule out such a possibility, based on qualitative aspects of the antigen, I immunized with a 'low' dose of SRBC-OVA, lethally irradiated mice that had been reconstituted with either $2x10^7$ normal spleen cells alone, or with this number of normal spleen cells and in addition, increasing numbers of transgenic spleen cells, beyond the number needed to generate a Th1 response. When more transgenic spleen cells are given than the $1x10^6$ found to be required to support the generation of Th1 cells, substantial numbers of anti-SRBC Th2 cells are generated, see Figure 10. These observations support the hypothesis that more CD4 T cell cooperation is required to generate Th2 than Th1 cells (Ismail and Bretscher 1999). In the following sub-section, the populations involved in, and the details of the mechanism of these cooperative events will be addressed.

Figure 10: Increasing numbers of DO11.10 spleen cells can progressively support the generation of both IFN-γ and IL-4-producing anti-SRBC cells

Lethally irradiated mice were reconstituted with either $2x10^7$ normal spleen cells alone (normal) or with an additional population of spleen cells from a naive DO11.10 donor, as indicated (% DO11.10). With the exception of unchallenged controls (✶), all mice were immunized with 200 μL of .01% SRBC-OVA. The number of SRBC-dependent, IFNγ (white) and IL-4 (black) producing cells per 10^6 input unprimed spleen cells was assessed on day 7 post-immunization. Responses for individual mice are shown. These observations represent two separate experiments. Similar results were seen when CRBC was employed as antigen (not shown).





note: 'γ' symbols represent reconstituted, unimmunized controls

5.4 The Threshold hypothesis predicts that CD4 T cells are critical in both the induction and effector phases of the immune response

The Threshold Hypothesis predicts that one critical variable in determining the Th1/Th2 phenotype of effector CD4 T cells generated is the precursor frequency of antigen-specific CD4 T cells before challenge. This relationship between the precursor T helper cell number and the nature of the effector CD4 T cells generated, forms the basis of the teleological framework behind the decision criterion proposed in this model, which in large part separates it from other models of Th1/Th2 differentiation, as discussed earlier. It was therefore necessary to test, in this experimental system, whether precursor CD4 T cells, present in the DO11.10 spleen, affected the phenotype of XRBC-specific responding CD4 T cells, present in reconstituting normal splenic population. While a similar relationship has already been demonstrated in a related adoptive transfer system employing XRBC as antigen (Ismail and Bretscher 2001; Ismail, 2000), the use of transgenic CD4 T cells in this system represents a significant difference, warranting confirmation of this relationship, and can also lead to a more incisive analysis of mechanism.

5.4.1 The cells obtained from the spleens of lethally irradiated, reconstituted, and immunized mice producing antigen-dependent cytokines are CD4 T cells

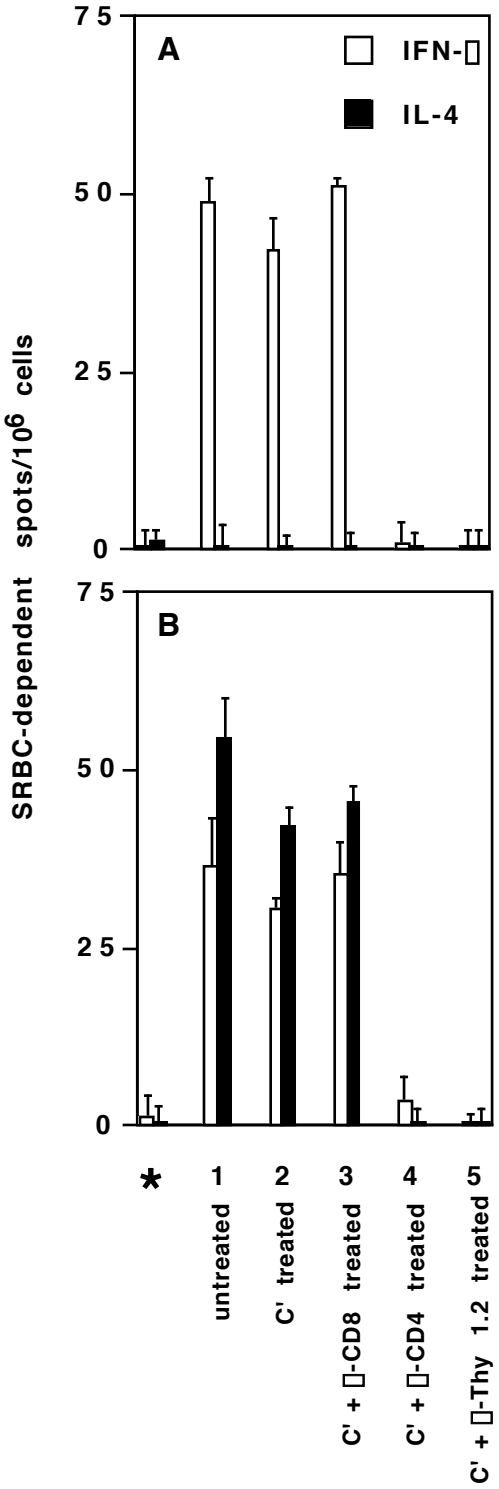
To determine which cells are responsible for the production of SRBC-specific cytokines in the ELISPOT assay during the effector phase of the immune response, cells from the spleens of reconstituted and immunized mice, taken 7 days after immunization, were depleted of various cellular subsets before being plated in the ELISPOT assay. Figure 11 shows that CD4 T cells are responsible for both SRBC-specific IFN-γ and IL-4 production. As expected, the presence of DO11.10 spleen cells in the reconstituting population (Figure 11b) did not cause an additional or alternate population of spleen cells to produce antigen-dependent IFN-γ or IL-4. As mentioned, these results are in agreement with previous adoptive transfer studies employing XRBC as antigen.

5.4.2 The critical cell in the DO11.10 spleen, able to modulate the XRBC-specific Th1/Th2 response phenotype is a CD4 T cell, as determined by antibody and complement depletion

In order to verify that antigen-specific CD4 T cell frequency is critical in determining the specific Th1/Th2 phenotype, DO11.10 spleen cells were depleted of CD4 bearing cells before their use as a 'spiking' reconstituting population of cells for

Figure 11: Phenotypic characterization of the XRBC-dependent spot-forming cell seen in the ELISPOT assay, as assessed by antibody and complement depletion

Lethally irradiated BALB/c mice were reconstituted with $2x10^7$ unprimed normal spleen cells alone (**A**) or with $2x10^7$ unprimed normal spleen cells and $1x10^6$ unprimed, untreated, spleen cells from a DO11.10 donor (**B**). With the exception of unchallenged control mice (✶), experimental mice were immunized with 200 μL of 0.1% SRBC-OVA. On day 7 post-immunization, spleens from three mice per group were obtained, processed, and pooled. Effector cells were either not treated (group **1**), treated with complement (group **2**), treated with complement and anti-CD8 depleting antibody (group **3**), treated with complement and anti-CD4 depleting antibody (group **4**), or treated with complement and anti-Thy 1.2 depleting antibody (group **5**). The number of SRBC-dependent IFNγ (white bars) and IL-4 (black bars) producing cells per 10^6 input unprimed spleen cells +/- SD was assessed using the ELISPOT assay. These observations are a single representative of three similar experiments.



note: 'α' symbol represent reconstituted, unimmunized controls

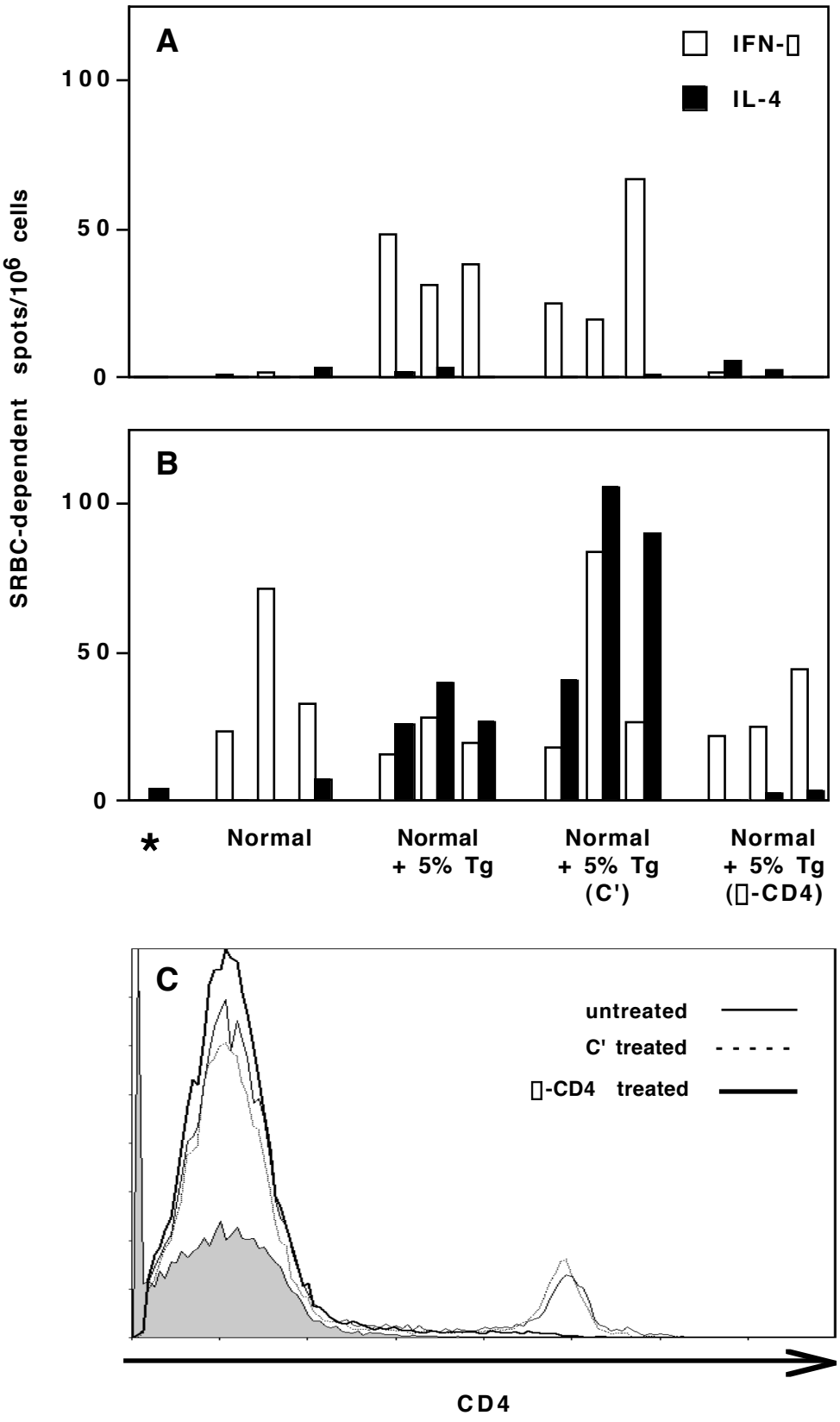
lethally irradiated mice immunized with SRBC-OVA. As seen in Figure 12 a and b, when DO11.10 spleen cells are depleted of CD4+ cells, the effect of the additional 'spike' of transgenic cells is lost. This result is independent of antigen dose. The effectiveness of depletion of CD4+ cells T cells was verified by FACS analysis of CD4 bearing cells (Figure 12c), and a similar pattern (over 90% depletion) was observed with analysis using the anti-OVA-specific TCR clonotypic antibody (not shown). CD8 T cell numbers in CD4 T cell-depleted samples of DO11.10 spleen cells would not be expected to be dramatically increased, as a very small number (~2%) are present in unmanipulated DO11.10 spleen (not shown).

5.4.3 The critical cell in the DO11.10 spleen able to modulate the XRBC-specific Th1/Th2 response is a CD4 T cell, as determined by MACS sorting

While the results presented in Figure 12 provide strong evidence as to the importance of CD4 T cells in affecting the Th1/Th2 phenotype of an immune response, perhaps a more elegant approach in showing a decisive role for CD4 T cells cooperation in affecting Th1/Th2 phenotype is represented by using purified OVA-specific CD4 T cells to 'spike' reconstituting populations given to mice challenged with SRBC-OVA. Another potential reason to pursue this approach is because the depletion of CD4 T cells from DO11.10 spleen cells before their use as reconstituting populations could dramatically affect the relative proportion of other large and small spleen cell populations (for example CD8 T cells) in the

Figure 12: The critical Th1/Th2 phenotype-modulating cell in the DO11.10 spleen is a CD4(+) T cell, as assessed by antibody and complement depletion

Lethally irradiated mice were reconstituted with $2x10^7$ unprimed normal spleen cells alone or with an additional $1x10^6$ untreated DO11.10 spleen cells (normal + 5% Tg), with $1x10^6$ complement treated DO11.10 spleen cells (normal + 5% Tg C'), or with $1x10^6$ complement and anti-CD4 antibody treated DO11.10 spleen cells (normal + 5% Tg □-CD4). With the exception of unchallenged controls (✶), mice were immunized with 200 □L of either .01% (**A**) or .1% (**B**) SRBC-OVA. The number of SRBC-dependent IFN□ (white) and IL-4 (black) producing cells per 10^6 input unprimed spleen cells was assessed 7 days post-immunization. Responses for individual mice are shown. (**C**) Samples of untreated (light line), complement treated (broken line) and □-CD4 treated (clone GK 1.5) (heavy line) DO11.10 spleen cells were stained with fluorescence-tagged anti-CD4 antibody (clone RM4-4, recognizing a different epitope than clone GK 1.5) and analyzed to confirm the effectiveness of CD4 depletion. These observations are a representative of two separate experiments.

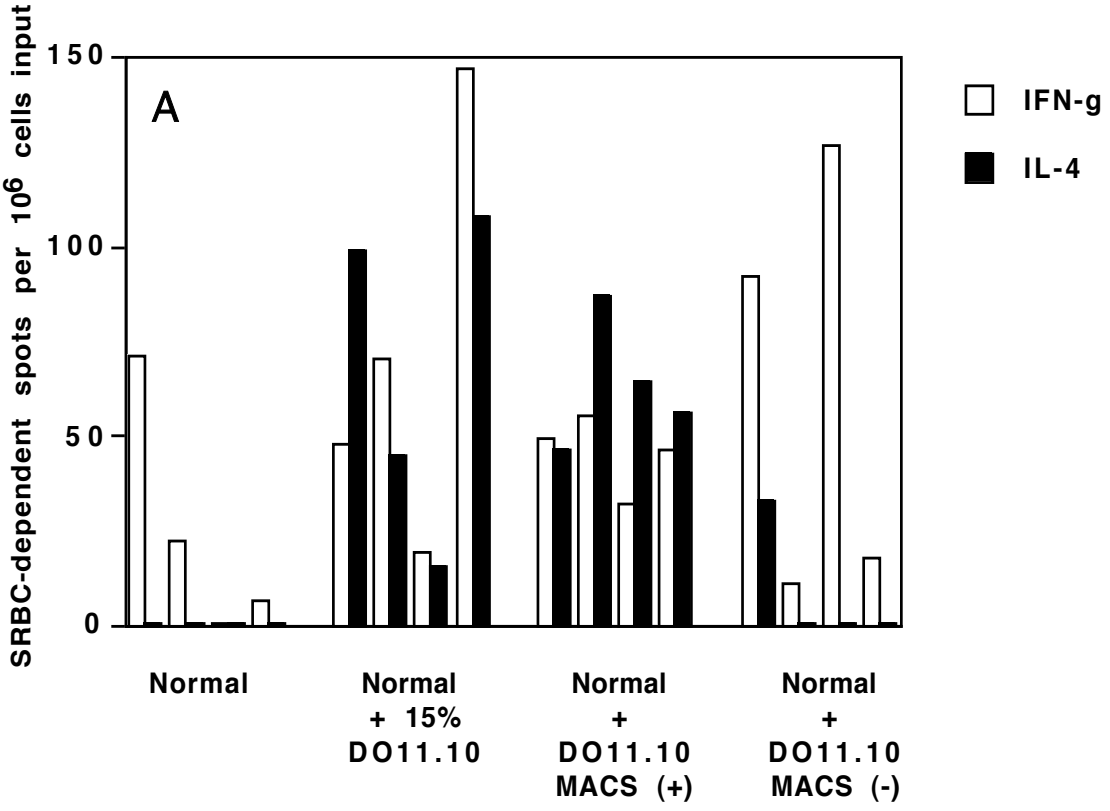

A
IFN-γ
IL-4
100
50
0
B
100
50
0
SRBC-dependent spots/10^6 cells
*
Normal
Normal + 5% Tg
Normal + 5% Tg (C')
Normal + 5% Tg (α-CD4)
C
untreated
C' treated
α-CD4 treated
CD4

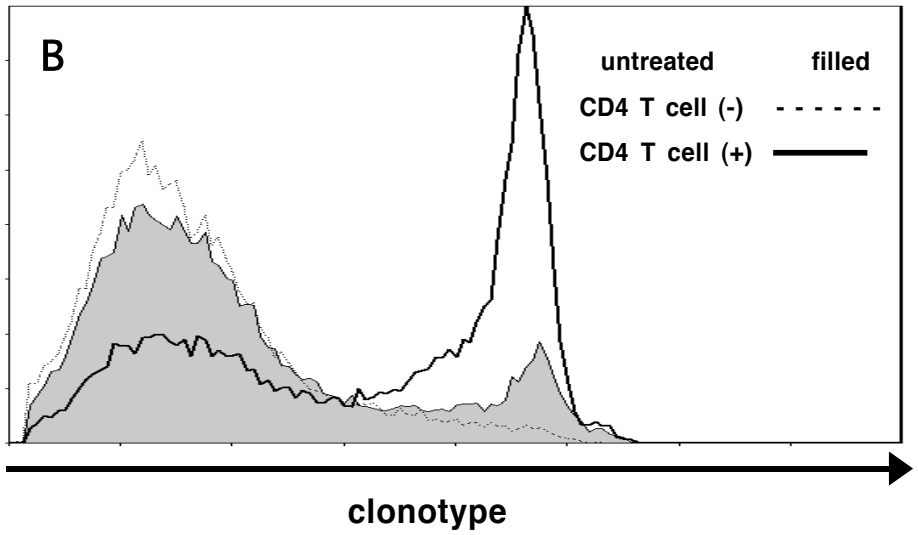
reconstituting population, potentially influencing the results observed. To isolate enriched populations of transgenic CD4 T cells, I employed MACS sorting – specifically, a procedure to obtain "untouched" CD4 T cells (by negative selection, see Materials and Methods).

As seen in Figure 13a, reconstitution with normal spleen cells and a population of MACS purified CD4 T cells (MACS+) generated SRBC-specific responses with a substantial Th2 component, while reconstituting with the same number of positively selected, CD4 T cell depleted DO11.10 spleen cells (MACS-) did not. Importantly, in this experiment, mice were reconstituted with $2x10^7$ normal spleen cells and with either *15%* untreated DO11.10 spleen cells ($3x10^6$), or with a population of MACS sorted DO11.10 cells roughly representing the number of CD4 T cells present in $3x10^6$ spleen cells – roughly 15% or $4.5x10^5$ cells. I chose to employ 15% populations of transgenic donar cells in this experiment because I felt that the number of MACS-purified CD4 T cells that would represent a 5% 'spike' ($1.5x10^5$), might be difficult to transfer accurately, potentially becoming limiting through slight pipptetting or other experimental errors. MACS sorting was verified by FACS analysis of TCR clonotype bearing cells (Figure 13b) and a similar pattern was observed with analysis with anti-CD4 (not shown). MACS sorted CD4 T cells were always over 90% pure when analyzed with either CD4- or clonotype-specific antibodies, and this purity was similar to MACS sorting employing positive selection of CD4 T cells (used in other experiments).

Figure 13: The critical Th1/Th2 phenotype-modulating cell in the DO11.10 spleen is a CD4 (+) T cell, as assessed by MACS separation

Lethally irradiated mice were reconstituted $2x10^7$ unprimed normal spleen cells alone (Normal) or with an additional $1x10^6$ untreated DO11.10 spleen cells (Normal + 15% Tg), or with $2x10^7$ normal spleen cells and a number of cells from the CD4 T cell (+) or (-) MACS fractions representing the number of CD4 T cells present in $3x10^6$ DO11.10 spleen cells (based on previous FACS analysis). With the exception of unchallenged controls (✶), mice were immunized with 200 μL of .01% SRBC-OVA. The number of SRBC-dependent IFNγ (white) and IL-4 (black) producing cells per 10^6 input unprimed spleen cells was assessed 7 days post-immunization. Responses for individual mice are shown. (**B**) Samples of untreated (filled), MACS CD T cell (-) fraction (open, broken line) and MACS CD4 T cell (+) fraction (open, heavy line) DO11.10 spleen were stained with FITC-conjugated anti-clonotypic antibody and analyzed to confirm the effectiveness of CD4 depletion. Similar results were obtained when representative samples were analyzed for CD4 expression (not shown). These observations are a representative of two similar experiments.


A
SRBC-dependent spots per 10^6 cells input
150
100
50
0
IFN-g
IL-4
Normal
Normal + 15% DO11.10
Normal + DO11.10 MACS (+)
Normal + DO11.10 MACS (-)


B
untreated
filled
CD4 T cell (-)
CD4 T cell (+)
clonotype

5.4.4 Cooperation between CD4 T cells specific for OVA and CD4 T cells specific for XRBC is facilitated by the recognition of linked antigenic determinants

Data summarized to this point in this sub-section supports a model in which cooperative events between antigen-specific CD4 T cells during the induction phase of an immune response influence the Th1/Th2 cytokine secretion profile of CD4 T cells active in the effector phase of the response. There are several possibilities as to the particular form of, and functional mediators of this type of CD4 T cell cooperation. The following experiments were carried out to investigate important aspects of the cooperative mechanism between CD4 T cells, affecting the Th1/Th2 phenotype of the response generated.

Observations of independence between concurrent, relatively polarized Th1 and Th2 antigen-specific responses suggest that the Th1/Th2 decision making is rather localized. While it is easier to envisage several possible mechanisms which would provide independence of Th1/Th2 responses in situations where different antigens are administered via different routes of immunization, concurrent independent Th1/Th2 responses can be generated in the same lymphoid organ (Ismail and Bretscher, 2001) suggesting a more limited number of possibilities. For example, it is unlikely that long-range, polarizing cytokines (see 1.10.1) are alone responsible, especially when considering that a primary Th1 or Th2 response can be

generated in a lymphoid organ during the peak response period of an opposite, polarized response (Ismail, 2000).

This suggests a very intimate association of antigen-specific CD4 T cells and an APC bearing peptides derived from the nominal antigen (as murine CD4 T cells themselves do not express MHC II and are therefore unlikely to cooperate directly). The intimate association of only *antigen-specific* CD4 T cells around a given APC is easiest explained if the critical APC mediating the CD4 T cell cooperation, that determines the Th1/Th2 phenotype, displays epitopes derived from only one nominal antigen at a time. A more comprehensive presentation of this model, and its implications, can be found in the discussion. Testing this hypothesis simply, mice reconstituted with $2x10^7$ normal spleen cells and 5% DO11.10 spleen cells were challenged with either a high dose of SRBC-OVA, or the same dose of tannic acid treated SRBC and soluble OVA protein. As expected, mice receiving the conjugate antigen responded with a strong Th2 component, whereas mice immunized with uncoupled SRBC did not (data not shown). This experimental design is not ideal however, as it has long been known that soluble proteins can induce a state of tolerance, which can also explain these results independently of our hypothesis.

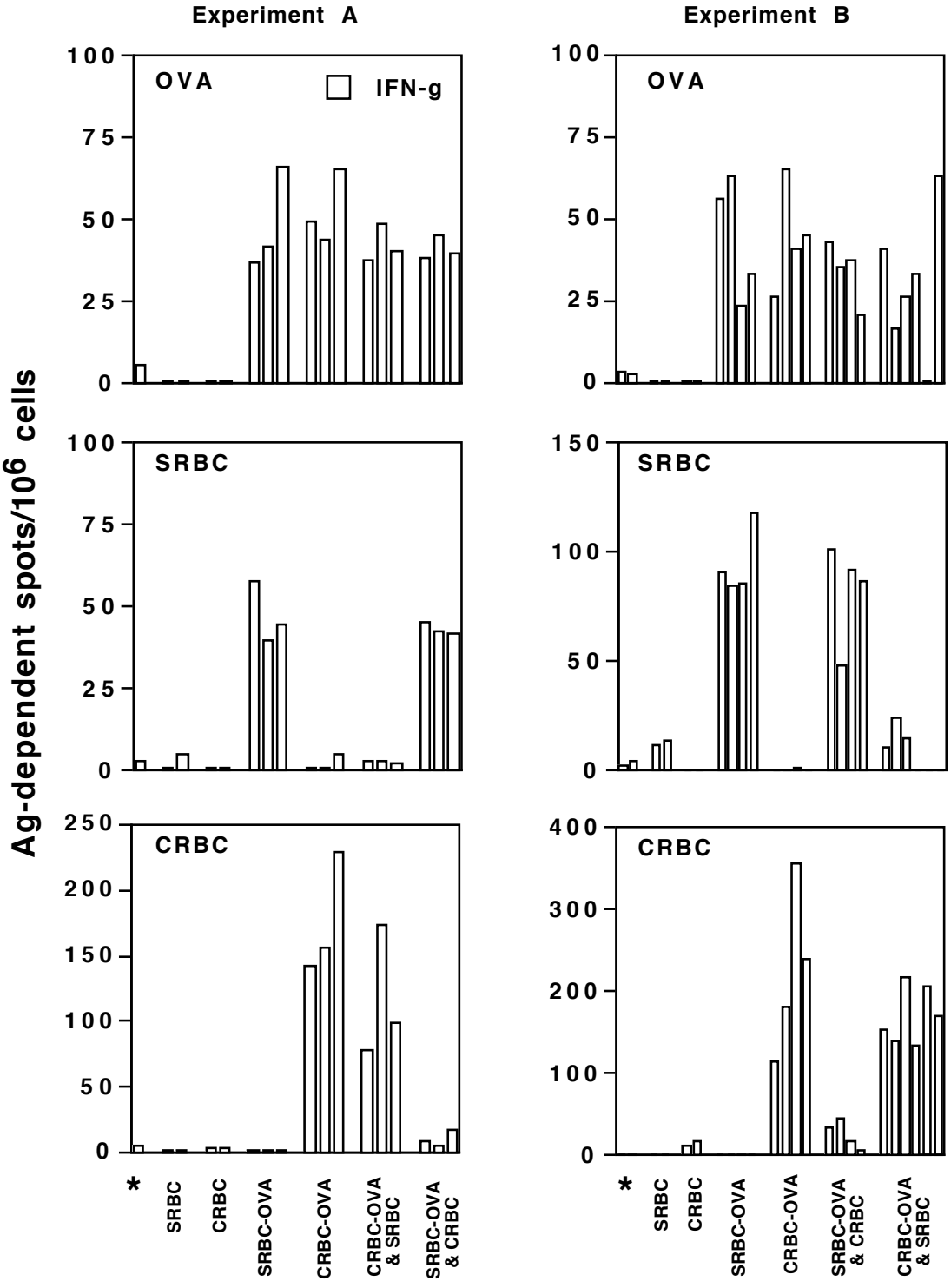
Therefore, I next examined in a far more physiologically relevant manner, whether the influence of OVA-specific transgenic CD4 T cells on the Th1/Th2 phenotype of the anti-XRBC response required physical linkage of XRBC to OVA. I reconstituted mice with $2x10^7$ normal and $1x10^6$ DO11.10 spleen cells, and immunized with a dose of either SRBC-OVA or CRBC-OVA that is sub-immunogenic in such reconstituted animals, based on preliminary experiments (see

5.3.1). In addition, and critically, both RBC were given to the same mouse simultaneously, but with OVA conjugated to only one of the XRBC populations. It is evident from Figure 14 that OVA-specific CD4 T cells only enhance the anti-XRBC response of the RBC to which OVA is physically coupled. It is noted that in some mice receiving both populations of XRBC, a slight cross-reactive effect is seen, perhaps reflecting some low level of peptide cross-reactivity in these BALB/c mice.

A similar dependency for 'linked' antigenic determinants has been reported in an experimental system using plasmids coding for antigens: mice were found to generate responses against an "immunologically silent" tumor antigen only if the plasmid coding for this antigen also coded for a segment of the *Plasmodium falciparum* circumsporozoite antigen (Gerloni et al., 2000). Injecting mice with separate plasmids (but in a single inoculum), each coding for an individual antigen, resulted in greatly diminished responses – roughly 15% of the response generated against the tumor antigen when mice were immunized with the plasmid encoding both antigens. When both separate antigen encoding plasmids were used to immunize mice, but injected at separate sites, the response generated against the tumor antigen (as measured by antigen-dependent IL-2, IL-4, IFN-γ, and T cell proliferation) was "entirely abrogated". A similar effect was seen in another experimental system using plasmid DNA, coated onto gold particles – presumably taken up by DCs - encoding either OVA or PCC to immunize naive mice also reconstituted with OVA or PCC TCR transgenic CD4 T cells (Creusot et al., 2003). In mice reconstituted with a small number of both transgenic CD4 T cell

Figure 14: OVA-specific CD4 T cells can support the generation of anti-XRBC IFN-γ-producing CD4 T cells only when OVA and the XRBC are linked (XRBC-OVA)

All lethally irradiated mice were reconstituted with $2x10^7$ normal spleen cells and $1x10^6$ DO11.10 spleen cells. With the exception of unimmunized controls (✶), mice were immunized with 100μL of 0.02% SRBC, CRBC, SRBC-OVA, CRBC-OVA alone, or with 100 μL of 0.02% each XRBC-OVA conjugate and 100 μL of 0.02% alternate uncoupled XRBC (as indicated). Importantly, the dose of each XRBC or XRBC-OVA conjugate was equivilant to 200μL of 0.01% antigen. The number of OVA-, SRBC-, and CRBC-dependent IFN-γ-producing cells per 10^6 input unprimed spleen cells was assessed 7 days post immunization. Responses for individual mice are shown. In all cases, IL-4 spots were negligible, and are not depicted. Results from two separate experiments are shown. Note the differences in scale between panels and between experiments. Also, note the differences in number of mice per group between experiments.


Experiment A
Experiment B
OVA
IFN-g
SRBC
CRBC
Ag-dependent spots/10^6 cells
100
75
50
25
0
150
250
200
400
300
*
SRBC
CRBC
SRBC-OVA
CRBC-OVA
CRBC-OVA & SRBC
SRBC-OVA & CRBC
note: '□' symbols represent reconstituted, unimmunized controls

populations, a cooperative effect was seen (enhanced antigen-specific responses) in mice immunized with both plasmids together, compared with responses seen in mice immunized with either plasmid alone, or with both plasmids, but given separately (on separate gold particle preparations).

This requirement for linkage of antigenic determinants can explain how the determination of the Th1/Th2 phenotype of simultaneous responses to non-crossreacting antigens can be independent, as the T cells specific for one of the two antigens will not cooperate with the CD4 T cells specific for other antigens. It also suggests that the functional mediators of CD4 T cell cooperation are extremely short-range in nature – perhaps a cytokine, perhaps a cell-surface molecule(s), or perhaps both.

5.4.5 Summary

In this section, I have presented experiments which show that the number of antigen-specific CD4 T cells present at the time of challenge can determine the Th1/Th2 phenotype of responding, effector CD4 T cells generated. Data supports the hypothesis that it is a cooperative mechanism whereby CD4 T cells present at the time of antigen challenge, in the induction phase, determine the general Th1/Th2 phenotype of antigen-specific cells. This CD4 T cell cooperation is mediated by the recognition of linked antigenic determinants (peptides) present on an APC, resulting in discrete 'decision-making circuits', which do not generally impact concurrent responses generated against non-cross-reacting antigens. These results support a

Th1/Th2 decision criterion as outlined in the Threshold Hypothesis. In the following section, I will describe experiments which attempt to further characterize the cellular populations and mechanisms involved.

5.5 A population of transgenic thymocytes is able to modulate the SRBC-specific Th1/Th2 response phenotype in a similar manner as a population of transgenic splenocytes when given to irradiated mice reconstituted with normal spleen cells and challenged with SRBC-OVA

Thymocytes, mature single-positive thymocytes, represent a source of T cells that are both less-antigen experienced than splenic T cells, and that are a more pure T cell population (with regards to other cellular populations present) than splenocytes. Several investigators have employed thymocytes as a population representing naive T cells (Pilarski, 1977, Strutt, 2005); evidence cited in the literature supports the use of thymocytes as a population of T cells representing functionally naive cells, as these cells have been found to respond against several stimuli in a manner similar to recent thymic emigrants, and express a phenotype similar to naive peripheral T cells (Fink et al., 1985; Ramsdell et al., 1991). Since the Threshold Hypothesis predicts that CD4 T cell *precursor* frequency (i.e., naive precursor T helper cells), can affect Th1/Th2 phenotype, and to further rule out any impact of previously activated T cells or APC on the Th1/Th2 decision, DO11.10 thymocytes were used in place of transgenic spleen cells as a 'spiking' population in mice reconstituted with normalBALB/c spleen cells and challenged with SRBC-OVA.

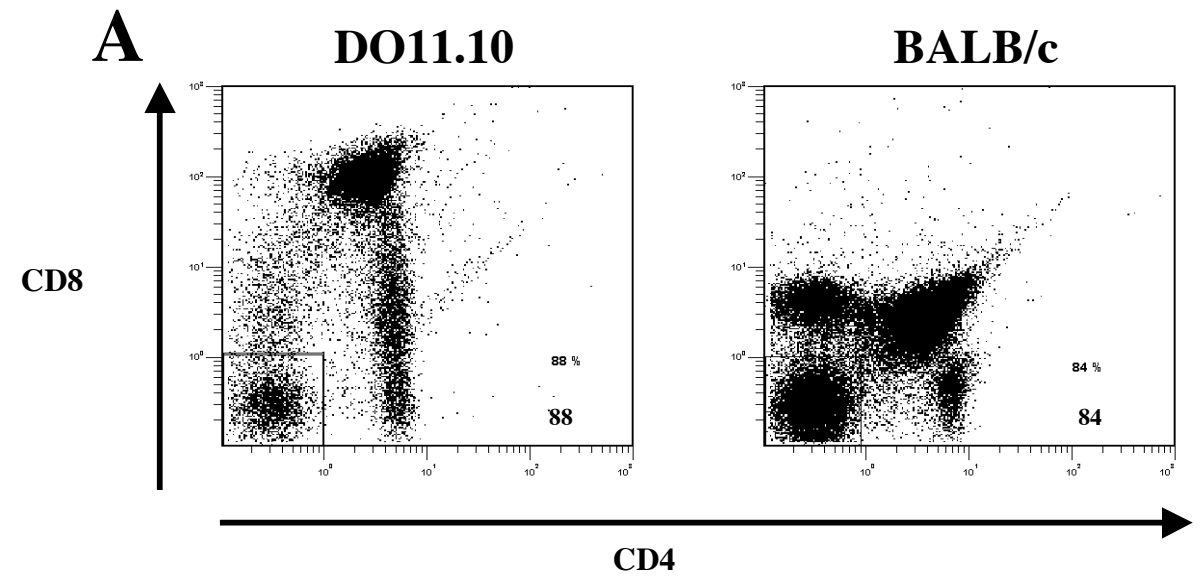
Importantly, thymocytes, while representing a relatively pure population of T cells, as mentioned above, represent a very heterogeneous T cell population based on

cell surface markers and maturation level (for example double positive CD4CD8 and double negative stages), and in significant contrast to a population of spleen cells when used as a reconstituting population, a large portion of these cells are not functional and appear destined to die (Benoist and Mathis, 1999). Thus, even though thymocytes represent a population of relatively pure T cells, the number of potentially functional, single-positive CD4 T cells in freshly isolated thymocyte populations is relatively low. Figure 15 presents FACS data obtained staining both BALB/c and DO11.10 thymocytes with either appropriately labled CD4 and CD8 or with CD19 and B220 antibodies.

Based on this reasoning, and on several pieces of FACS data comparing the number of single positive CD4 T cells present in the spleen and thymus of DO11.10 mice, and comparing the number of single positive CD4 T cells specific for OVA in normal spleen cell populations 'spiked' with 5% transgenic splenocytes or transgenic thymocytes (data not shown), I chose to employ the same percentage of thymocytes for 'spiking' as I previously did for DO11.10 spleen cells. As seen in Figure 16a, just as was seen with DO11.10 spleen cells, $1x10^6$ transgenic thymocytes can support the generation of a SRBC-specific Th1 response when given to irradiated mice reconstituted with $2x10^7$ normal spleen cells and challenged with a low dose of SRBC-OVA. Furthermore, increasing the number of transgenic thymocytes beyond that needed to generate a predominant Th1 response results in the generation of a significant anti-SRBC Th2 component, as characterized by the presence of antigen-dependent IL-4 producing T cells (Figure 16 a and b). These findings parallel those observed earlier with increasing numbers of transgenic spleen cells (see 5.3.7).

Figure 15: A comparison between thymic CD8/CD4 double-positive and CD19/B220 double-positive populations in age-matched DO11.10 and BALB/c mice

Age matched, naive, female DO11.10 and BALB/c mice were sacrificed, their thymuses removed, and samples prepared for FACS analysis, using relevant PE- (CD8 or CD19) and FITC- (CD4, B220) conjugated antibodies, as described (controls not shown). The rough percentage of T cell marker-positive cells is given for both DO11.10 and BALB/c mice (**A**) as is the percentage of non-staining, CD19/B220 double-negative cells (**B**).


A
DO11.10
BALB/c
CD8
88 %
88
84 %
84
CD4

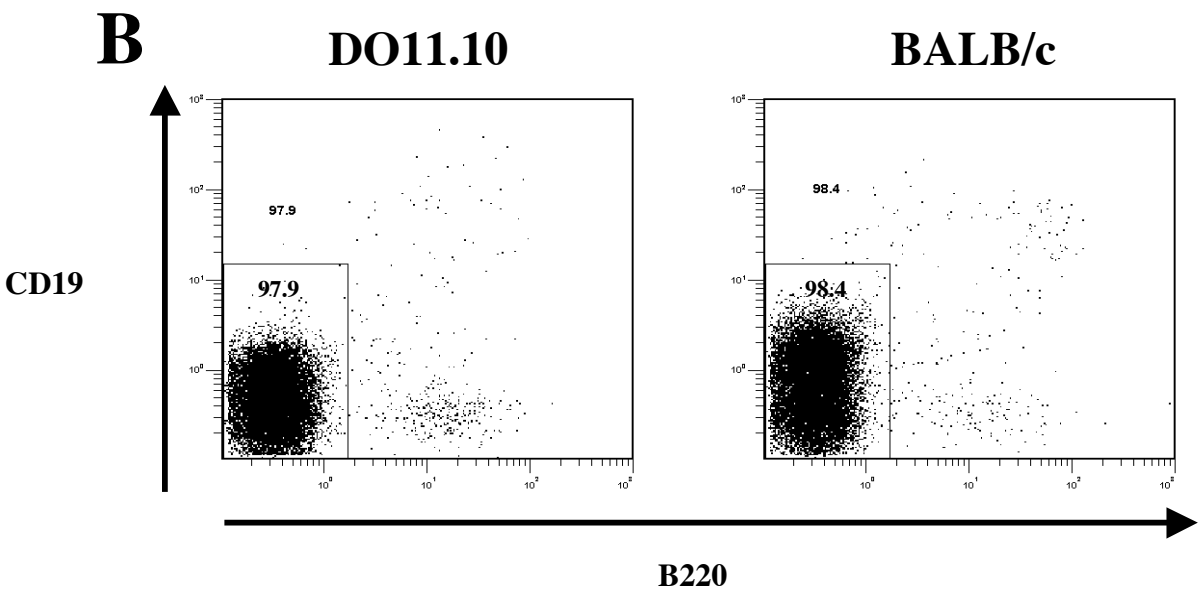
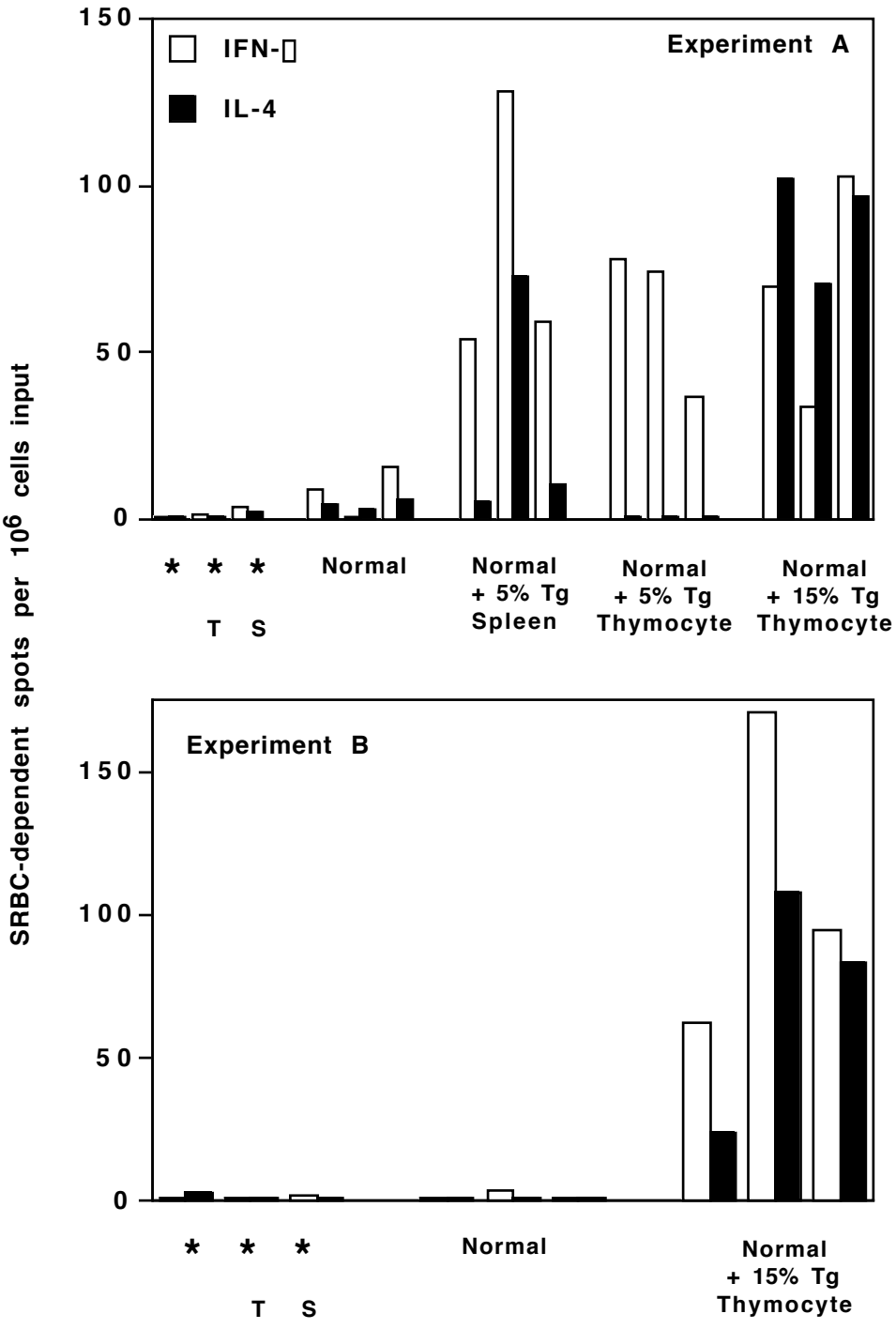

B
DO11.10
BALB/c
CD19
97.9
97.9
98.4
98.4
B220

Figure 16: OVA-specific, transgenic thymocytes can support the generation of both IFN-γ and IL-4-producing anti-SRBC cells in a pattern similar to that observed with transgenic splenocytes

Lethally irradiated mice were reconstituted with either $2x10^7$ normal spleen cells alone (normal) or with an additional population of spleen cells or thymocytes from a DO11.10 donor, as indicated (% Tg). With the exception of unchallenged controls (✶, reconstituted with either no additional transgenic cells, with 5% transgenic spleen cells [S] or 5% transgenic thymocytes [T]), mice were immunized with 200 μL of .01% SRBC-OVA. The number of SRBC-dependent IFN-γ (white) and IL-4- (black) producing cells per 10^6 input unprimed spleen cells was assessed 7 days post-immunization. Responses of individual mice are shown. These observations represent two of four separate experiments.



note: ' * ' symbols represent reconstituted, unimmunized controls

These results support a Th1/Th2 decision criterion involving antigen-mediated cooperation of, and subsequent activation of, naive CD4 T cells.

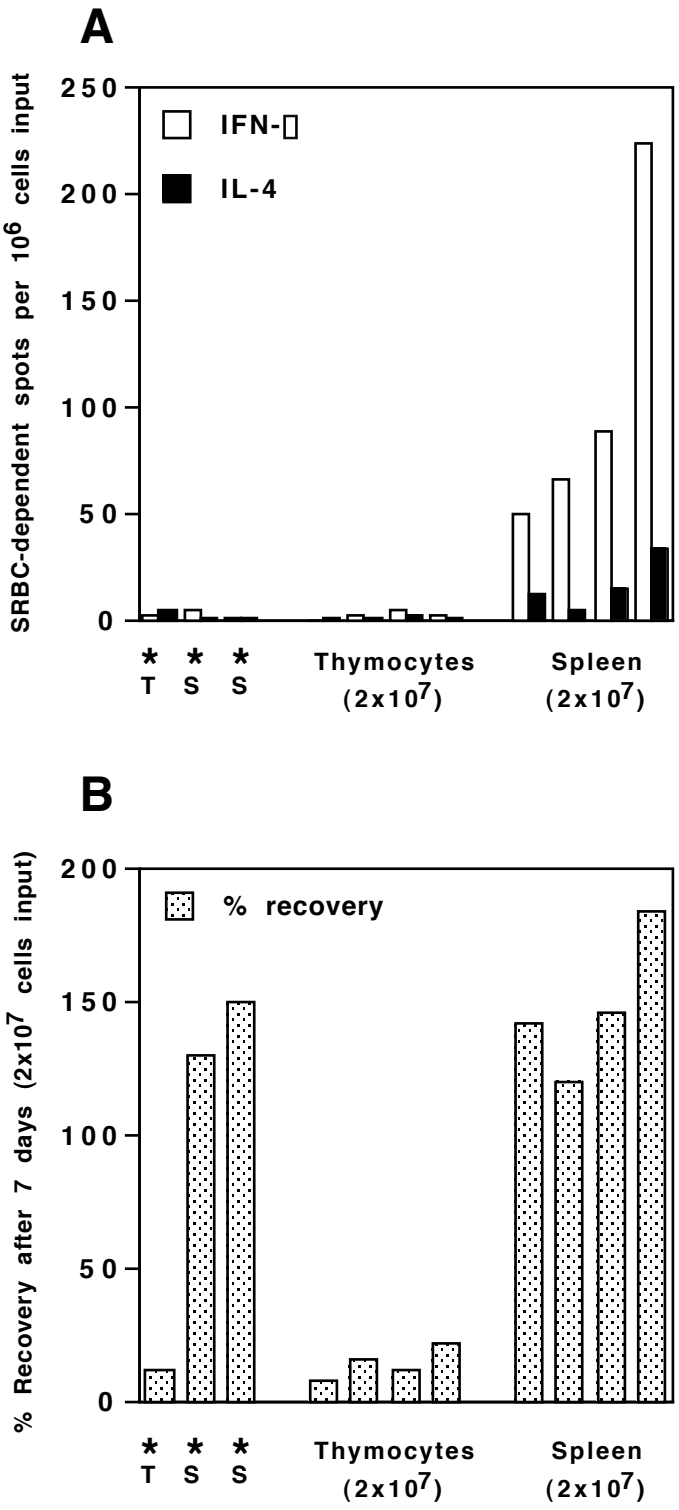
5.5.1 BALB/c thymocytes are not able to generate a significant SRBC-specific response when used to reconstitute lethally irradiated mice challenged with SRBC-OVA

I wished to test whether the population of normal BALB/c spleen cells, used to reconstitute lethally irradiated mice challenged with antigen, could also be replaced by an equivalent number of syngeneic thymocytes. A system in which the antigen-specific Th1/Th2 phenotyoe could be varyied by the reconstituting number *of both* XRBC-specific and OVA-specific thymocytes would strengthen the position that cooperation between naïve CD4 T cells is an important variable in determining the phenotype of a primary immune response. In experiments comparing mice reconstituted with populations of either BALB/c thymocytes or BALB/c splenocytes and challenged with a low dose of SRBC-OVA, no anti-SRBC responses were generated by irradiated mice receiving only thymocytes (Figure 17a). Furthermore, the recovery of cells from the spleen of mice reconstituted with thymocytes was dramatically reduced compared to recoveries of mice reconstituted with normal spleen cells, and this trend in recovery was not dependent on antigen (Figure 17b).

To rule out the possibility that $2x10^7$ thymocytes contain too few functional CD4 T cells to generate a SRBC-specific response when challenged with limiting amounts of antigen, and because some older observations suggested that

Figure 17: A population of normal thymocytes cannot generate substantial numbers of cytokine-producing anti-SRBC cells when used to reconstitute lethally irradiated mice challenged with SRBC-OVA

Lethally irradiated mice were reconstituted with either $2x10^7$ normal spleen cells or with $2x10^7$ normal thymocytes. With the exception of unchallenged controls (✶, reconstituted with either normal spleen cells (S) or thymocytes (T), mice were immunized with 200 μL of .1% SRBC-OVA. The number of SRBC-dependent IFNγ (white) and IL-4 (black) producing cells per 10^6 input unprimed spleen cells was assessed 7 days post-immunization (**A**). The recovery of spleen cells at day seven is represented as a percentage of initial input (**B**). These results are a single example of seven similar experiments. Responses for individual mice are shown.



note: '□' symbols represent reconstituted, unimmunized controls

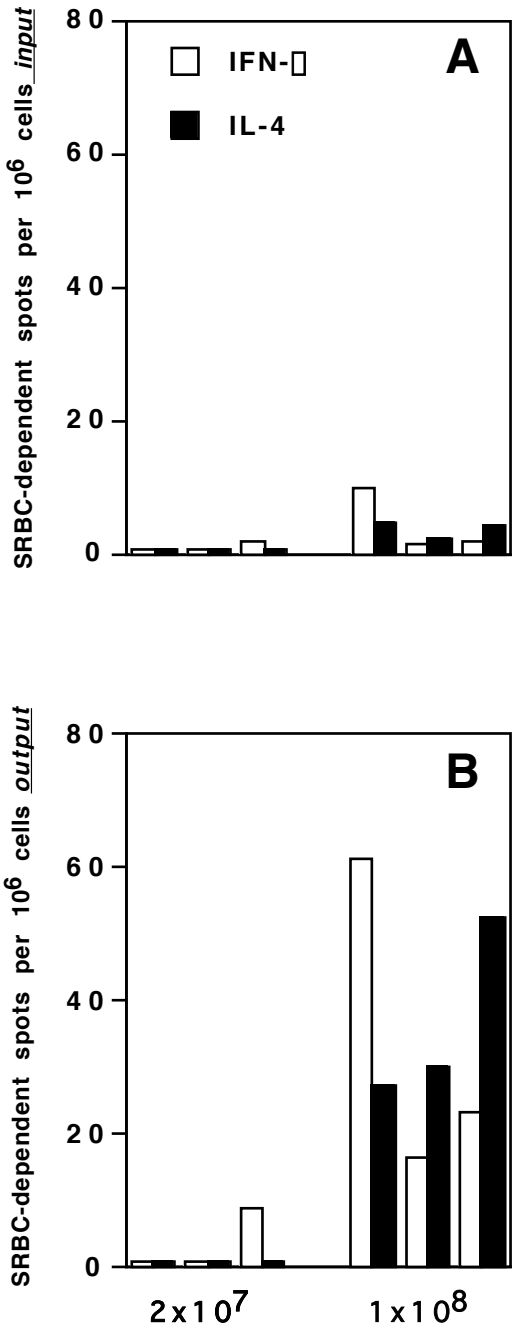
reconstitution with higher numbers of thymocytes can result in the generation of substantial immune responses (P. Bretscher, personal communication), lethally irradiated mice were reconstituted with either $2x10^7$ or with $1x10^8$ thymocytes and were challenged with a high dose of SRBC-OVA. As they did in response to a low dose of antigen, mice reconstituted with $2x10^7$ thymocytes and challenged with a high dose of antigen did not generate a significant SRBC-specific immune response (Figure 18). Mice receiving $1x10^8$ thymocytes did generate a significant number of raw, antigen-dependent spots, confirming that thymocyte reconstitution can result in functional immune responses (Figure 18b). However, the recovery of cells from the spleens of these mice was equally low when compared to mice receiving $2x10^7$ thymocytes (data not shown), resulting in a barely detectable normalized response (Figure 18b). This parameter reflects an extremely inefficient generation of effector CD4 T cells in irradiated mice reconstituted with $1x10^8$ thymocytes.

Together, these results suggest that some population(s) of cells, present in the naive splenic population of BALB/c mice, but not in the thymic population, is important in the activation and/or Th1/Th2 differentiation of antigen-specific CD4 T cells.

The following experiments were undertaken to better characterize the population lacking in the reconstituting BALB/c thymic population. The Two Step Two Signal Model predicts that both an antigen-specific, preexisting effector CD4 T cell, and an antigen-specific B cell are potential cell types that might be limiting when only thymocytes are used to reconstitute irradiated hosts.

Figure 18: A high number of thymocytes ($1x10^8$) can generate a detectable SRBC-specific response upon challenge with SRBC-OVA, but inefficiently

Lethally irradiated mice were reconstituted with either $2x10^7$ or $1x10^8$ normal thymocytes and challenged with 200 μL of .1% SRBC-OVA. The number of SRBC-dependent IFNγ (white) and IL-4 (black) producing cells per 10^6 thymocytes was assessed 6 days post-immunization and is presented corrected per 10^6 cells *input* (**A**) or per 10^6 cells *recovered* (raw antigen-dependent spots) (**B**). Responses for individual mice are shown. The recoveries of cells from the spleens of all mice, regardless of the amount of thymocyte input was similar, generally under 25% (not shown).


A
IFN-γ
IL-4
SRBC-dependent spots per 10^6 cells input
80
60
40
20
0
B
SRBC-dependent spots per 10^6 cells output
80
60
40
20
0
2x10^7
1x10^8

5.5.2 A population of CD4 T cells with a surface marker indicative of an activated phenotype is detectable in irradiated BALB/c mice

The cellular populations present in the spleen, but not in the thymus of BALB/c mice, critical in generating a SRBC-specific immune response in this adoptive transfer system, could be either one of several APC populations, or an activated CD4 T cell. Both of these populations are required, in addition to a pool of naive, antigen-specific CD4 T cells and antigen, to generate an immune response, as envisioned by the Two-Step, Two-Signal Model, upon which the Threshold Hypothesis is based (Bretscher, 1999).

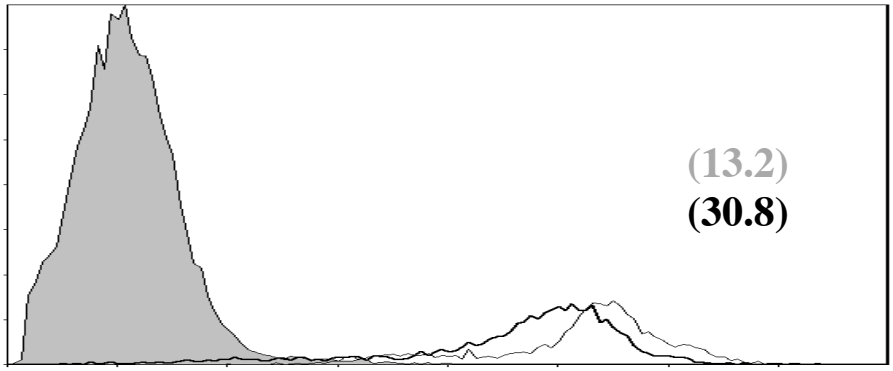
The critical 'helper' properties of activated CD4 T cells are radio-resistant (Pilarski, 1977, Strutt, 2005). Thus, if CD4 T cells with an activated phenotype persist in the irradiated host, then these cells might contain the effector cells necessary for the initiation of antigen-specific responses. To investigate this possibility, lethally irradiated mice were sacrificed one or two days after irradiation, their spleens removed, and analyzed by FACS for CD4 T cells displaying the activation marker CD44. The spleens of irradiated mice contained a relatively stable population of CD4/CD44 double-staining cells (Figure 19a). Much evidence suggests that such effector cells are relatively long lived *in vivo*, some at least up to 7 days after substantial doses of radiation (Williams et al., 1994; Agarossi et al., 1978). These data suggest that a population of helper CD4 T cells specific for SRBC or

Figure 19: A population of CD4 T cells persists in the spleen of lethally irradiated BALB/c mice two days after irradiation while the number of B cells falls dramatically

Naive BALB/c mice were either untreated (light line) or lethally irradiated with 850R and 2 days later (dark line), spleens were removed and stained with fluorescence-labeled antibodies as described above histograms. The relative populations of CD44/CD4 double positive **(A)** and B220/CD19 double positive **(C)** cells were determined by comparing either the CD44-positive cells in the CD4-positive gate, or comparing the CD19-positive cells in the B220-positive gate. Thy 1.2-positive cells (**B**) were determined by single-staining. Negative controls, to which no antibody was added are shown (grey); isotype controls (secondary antibody), which were similarly negative, are not depicted. This experiment was repeated employing CBA mice, with very similar results. The percentages of CD44 positive CD4 T cells (**A**), of Thy 1.2 positive (**B**), and of B220/CD19 double-positive (**C**) cells present in the spleens obtained from untreated, naïve mice (grey) and obtained from mice two days after lethal irradiation (black) is given in parentheses. The number of white blood cells recovered from the spleens of untreated mice was ~ $1x10^8$, the number of whilte blood cells recovered from the spleens of mice two days following lethal irradiation was ~ $1x10^7$ (ten-fold fewer).

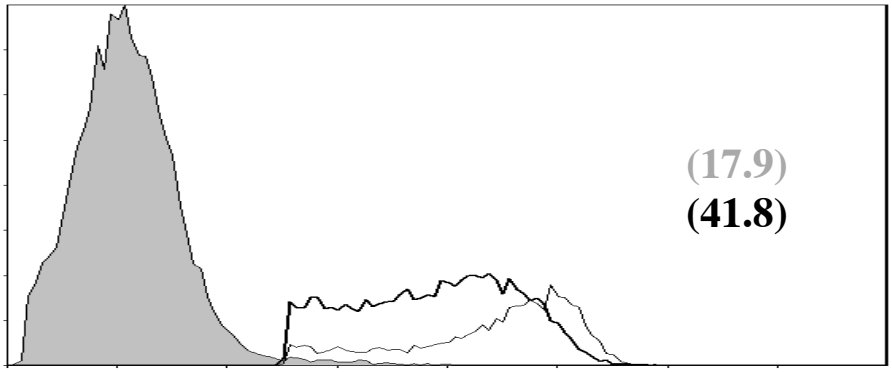
CD4 / CD44

A



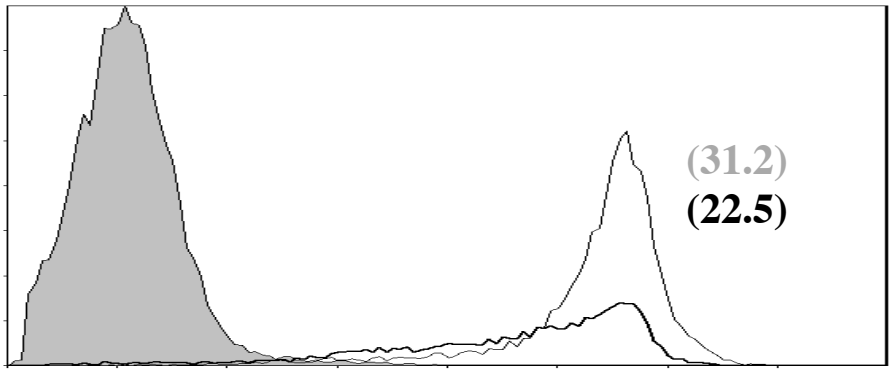
Thy 1.2

B



B220 / CD19

C



OVA, is perhaps not what is critically absent in the reconstituting thymocyte population, as a relatively stable population of effector CD4 T cells can be found in the spleen of irradiated hosts, and the proportion of Thy 1.2+ cells present in the irradiated host actually increases (Figure 19b) during the induction phase of an immune response.

The second possibility, that a source of APC is lacking in the thymocyte population, is supported by data presented in Figure 19c. While the proportion of CD4/CD44+ T cells in the spleen of irradiated mice is stable, or increases slightly two days after irradiation, the proportion and absolute number of B cells (B220/CD19+ cells) falls dramatically, as determined by FACS analysis. B cells were concentrated on in this experiment because of their envisaged importance in the Two Step Two Signal Model. If a limiting number of APC prevents reconstituting thymocytes from generating SRBC-specific responses – or even surviving after transfer - then the addition of a population of APC to the reconstituting thymocyte population should rescue an immune response. There is evidence in the literature suggesting that APC-dependent interactions are required for the short-term survival of CD4 T cells in an experimental system employing adoptive transfer of purified CD4 T cells into a relatively-lymphopenic environment (Martin et al., 2003).

5.6 A population of BALB/c T cell-depleted spleen cells, in conjunction with a population of BALB/c thymocytes is able to generate an SRBC-specific response

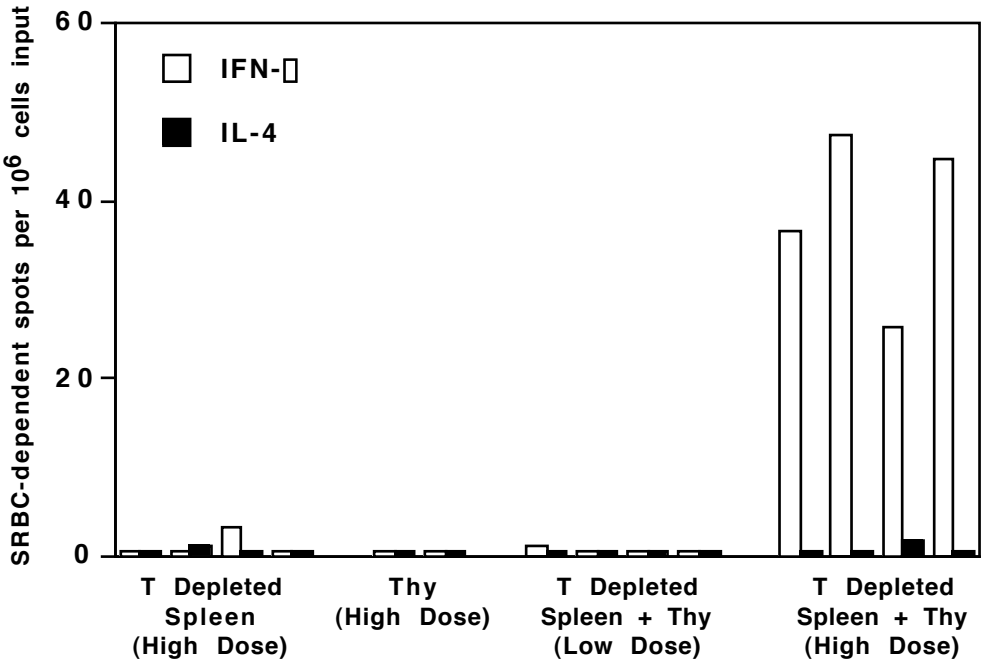
To test if thymocyte responses could be restored by the addition of APC, synergy was attempted between equal numbers of thymocytes and a population of naive BALB/c spleen cells depleted of T cells by antibody and complement treatment. While mice reconstituted with only $2x10^7$ thymocytes or with only $2x10^7$ T-depleted spleen cells and challenged with SRBC-OVA did not generate a SRBC-specific response, animals reconstituted with both populations generated a dose-dependent response, mirroring the pattern seen with reconstituting populations of whole spleen cells (Figure 20). The recovery of spleen cells from mice receiving whole spleen cells or T cell-depleted spleen and thymocytes was similar (data not shown). These results suggest a critical role for a population of, or several populations of, APC in the efficient activation of thymocytes.

5.6.1 A population of BALB/c MACS sorted CD4 T cell-negative spleen cells in conjunction with a population of BALB/c thymocytes is able to generate an SRBC-specific response

While T cell-depleted spleen cells, though not generating SRBC-specific cytokine-producing cells upon antigen challenge, supported the generation of anti-SRBC thymocyte responses, FACS analysis of T cell depletion of spleen cells was

Figure 20: A population of $2x10^7$ T cell-depleted BALB/c spleen cells and $2x10^7$ BALB/c thymocytes can synergize to generate a SRBC-specific response when such mice are challenged with SRBC-OVA

Lethally irradiated mice were reconstituted with either $2x10^7$ T cell-depleted spleen cells alone, $2x10^7$ thymocytes alone, or with both populations, and were challenged with 200 μL of .1% SRBC-OVA (high dose) or .01% SRBC-OVA (low dose). The number of SRBC-dependent IFNγ (white) and IL-4 (black) producing cells were assessed 6 days post-immunization and is presented as corrected per million responding (T cells) cells input, with the obvious exception of mice reconstituted with T cell-depleted spleen cells alone; responses in these mice were corrected per million cells input. This result is a single representitive of four similar experiments.


SRBC-dependent spots per 10^6 cells input
60
40
20
0
IFN-γ
IL-4
T Depleted Spleen (High Dose)
Thy (High Dose)
T Depleted Spleen + Thy (Low Dose)
T Depleted Spleen + Thy (High Dose)

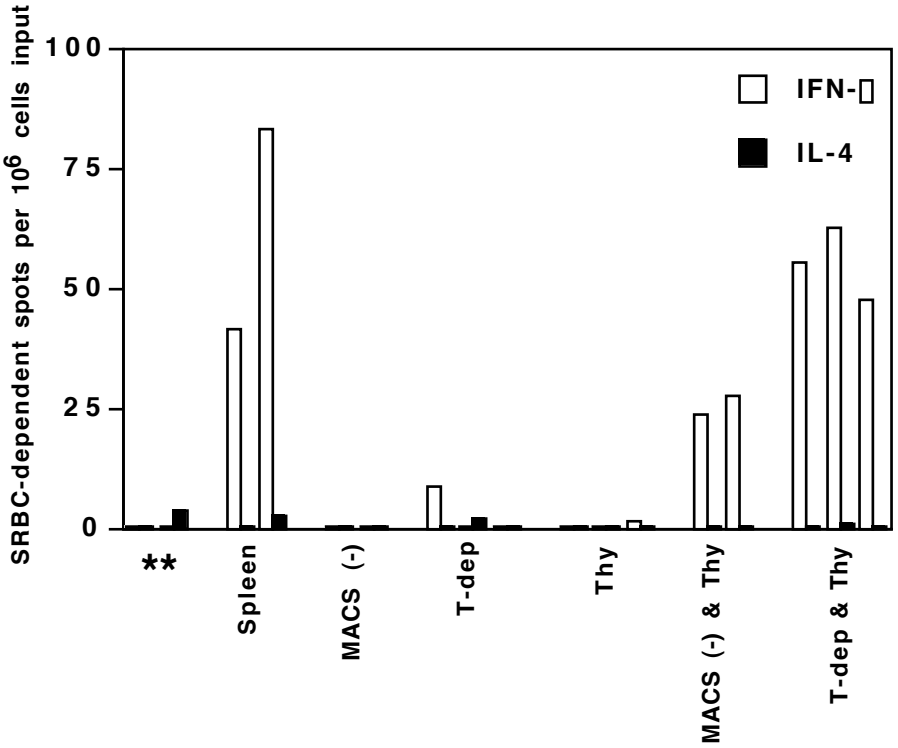
rarely above 90% and quite variable, in my hands. Thus, this finding cannot rule out the possibility that contaminating T cells, present in the T cell-depleted spleen cell population, and not APC populations, were responsible for 'rescuing' thymocyte responses. It should be kept in mind, however, that when used to reconstitute irradiated hosts alone, T cell-depleted spleen populations did not generate significant numbers of antigen-dependent cytokines (Figure 20). This finding suggests that though FACS analysis of T cell-depleted populations sometimes reveals a substancial population of contaminating CD4 T cells, this population might contain a number of CD4 T cells that are in the process of dying, or that are no longer functional. To obtain a 'cleaner' and more defined removal of CD4 T cells from splenic populations, I employed MACS sorting using a positively-selecting CD4 T cell isolation kit, which routinely yielded populations of negatively selected spleen cells with less than 5% contaminating CD4 cells. As can be seen in Figure 21, a population of CD4(-) MACS sorted spleen cells, while not responding to SRBC-OVA, did support the generation of SRBC-specific thymocyte responses, in a similar manner as did T cell-depleted spleen cells.

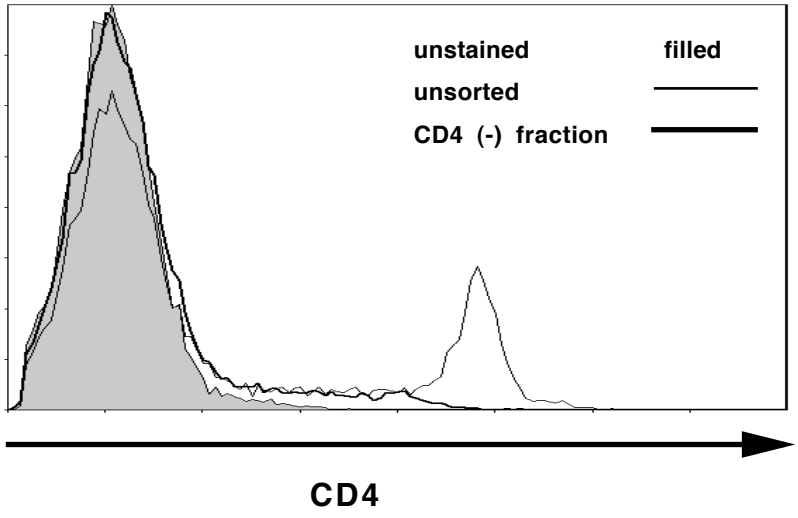
Combined, results employing reconstituting populations of T cell-depleted spleen cells and MACS-sorted CD4(-) spleen cells to complenent reconstituting thymocyte populations, suggest that APC present in the spleen but not in the thymus can 'rescue' antigen-specific immune responses generated by thymic CD4 T cells. This finding allows the potential to test whether a population of DO11.10, OVA-specific thymocytes can influence the Th1/Th2 phenotype of an XRBC-specific immune response when used to additionally reconstitute lethally irradiated hosts

challenged with XRBC-OVA and given BALB/c thymocytes and splenic APC. This experimental setup could be used to test questions first brought up in subsection 5.5.1, asking whether cooperative events between reconstituting thymocyte populations are able to influence Th1/Th2 phenotype in a manner similar to cooperative events between splenic T cells. If this were found to occur, it would strenthgen the hypothesis that cooperative, antigen-dependent signals can determine the Th1/Th2 nature of a primary immune response, as the reconstituting thymocyte populations (both XRBC- and OVA-specific) reflect populations of T cells not having experienced peripheral antigen encounter.

Figure 21: A population of CD4 T cell (-) MACS sorted spleen cells can facilitate SRBC-specific thymocyte responses in a similar manner as a population of T cell-depleted spleen cells

Lethally irradiated mice were reconstituted with either $2x10^7$ BALB/c spleen cells (spleen) $2x10^7$ T cell-depleted spleen cells (T-dep), $2x10^7$ CD4-negative MACS sorted spleen cells (MACS(-)), $2x10^7$ thymocytes (Thy) or combinations of these populations (as depicted). Except for unimmunized control mice (*), mice were challenged with 200 □L of .1% SRBC-OVA. The number of SRBC-dependent IFN□ (white) and IL-4 (black) producing cells per 10^6 thymocytes was assessed 7 days post-immunization and is presented corrected per million cells input (**A**). The efficiency of MACS sorting was verified by FACS analysis (**B**). Unsorted spleen cells (light line), the negatively selected CD4 negative MACS population (heavy line) and unstained sample of spleen cells is presented. A similar pattern was observed when samples were analyzed for the presence of double staining CD4/CD3 cells was done (not shown).





note: '□' symbols represent reconstituted, unimmunized controls

6.0 Discussion

6.1 Summary

The experimental evidence presented here confirms earlier studies showing that, as expected based on the Threshold Hypothesis, antigen dose can influence the Th1/Th2 phenotype of a primary immune response, when other variables are held constant. A further prediction of the Threshold Hypothesis supported by experimental findings presented here, is that when antigen concentration is held constant, increasing the number of antigen-specific CD4 T cells favors the generation of responses with a strong Th2 component in the presence of a relatively high dose of antigen, and when the amount of antigen is more limiting, then increasing numbers of antigen-specific CD4 T cells can 'rescue' a predominant Th1 response. These results suggest that the Th1/Th2 decision criterion can be impacted significantly by quantitative variables, and not only by qualitative differences between antigens, as predicted by other models.

In his veiled criticism of the German academia's inertia during the Nazi regime, *The Glass Bead Game*, Herman Hesse paints science (represented by the nation of Castilia) as a detached activity, outside of the realm of the everyday concerns and issues facing the general public. His protagonist, Joseph Knecht,

comes to realize the emptiness of the pursuit of knowledge for its own sake, deciding to abdicate his high position in Castilian society, and opting instead to offer what he can to his fellow citizens as a member of society at large. Knecht seeks, but tragically, does not find, an integration.

The pursuit of basic science, steeped in theory and mechanism, employing systems sometimes rather far removed from the natural condition, can sometimes make one sympathize with Hesse and his Knecht. However, in my particular situation, I am energized, and made to believe that these findings are beyond the purely academic, because of the many reports finding that predictions made by the Threshold Hypothesis are also valid when applied to animal models of human disease, employing replicating bacteria, pathogenic parasites, certain cancer cells, and viruses (Bretscher et al., 1992; Power et al., 1998; Uzonna and Bretscher, 2001; thoroughly reviewed in Bretscher et al., 2002). These studies not only justify, but compel research bearing on the more detailed mechanisms and inter-relationships between the variables of antigen dose and the number of responding CD4 T cells, affecting the Th1/2 response phenotype. The practical importance of such understanding, for example, as incorporated into the design of safe, effective, and relatively cheap vaccine strategies or immunotherapy regimes, represents the type of integration Hesse so strongly advocated (although Hesse, of course, was more disturbed by the silence and complacency of Germany's academic elite as related to social issues during the Third Reich) and the type of integration between basic research and practical application that Burnet felt, over 20 years ago, was no longer possible for the immunologist – prognosticating that it would be, "highly unlikely

that immunology-based ... procedures will be efficiently used in the treatment of such conditions as autoimmune disease" (Burnet, 1978). Burnet's essay describes immunology in similar terms as Hesse describes the Glass Bead Game – a purely intellectual pursuit with experimental findings and models that, "may come as a surprise to even the most sophisticated immunologists" (Bell and Perelson, 1978).

Pursuant towards such a goal of integrating basic theory and practical understanding, we have investigated several aspects of the interconnection between antigen dose and the number of antigen specific CD4 T cells, influencing immune response phenotype. By employing the conjugate antigen XRBC-OVA, and a source of OVA-specific CD4 T cells, it was shown that increasing numbers of antigen-specific CD4 T cells are able to, when added to a number of normal spleen cells and a dose of antigen that alone does not generate a detectable immune response, produce a predominant Th1 response. When added to an experimental setup leading to a Th1 response, additional transgenic cells can help in the generation of mixed Th1/Th2 antigen-specific responses.

These results suggest a cooperative mechanism involving antigen-specific CD4 T cells in determining the Th1/Th2 phenotype of the response generated, with optimal cooperation resulting in Th2 or humoral responses, and sub-optimal cooperation leading to largely Th1 or cell-mediated responses. Experiments presented in this thesis support a model of CD4 T cell cooperation between cells specific for different epitopes (peptides) only when these peptides are derived from the same nominal antigen. This cooperative restraint has often been referred to as 'linked recognition' or as the 'recognition of linked epitopes'. Importantly, the

finding of cooperation between T cells, limited to the recognition of linked epitopes, such as the OVA-specific CD4 T cells influencing the response generated against an OVA coupled RBC, but not the response generated against a concurrently administered, non-cross-reacting uncoupled RBC, suggests a further restraint on this cooperative mechanism. Namely, if the mediating signals involved in CD4 T cell cooperation were soluble, long-range factors, then independence between the responses against the OVA-coupled and uncoupled RBC might well be not so clearly observed – the *independence* seen between the responses supports a cooperative signal with very limited range, such as that mediated by the up-regulation of a cell-surface molecule or an extremely short-range cytokine. Finally, the general finding of *coherence* observed between the OVA-specific and XRBC-specific Th1/Th2 responses upon challenge with XRBC-OVA offers still more evidence in support of a model in which CD4 T cells specific for and responding against individual peptides derived from the same nominal antigen cooperatively influence and regulate each other's Th1/Th2 phenotype development.

Many pieces of data support the conclusion that a polarized cell or population of cells, present in the donor spleen, is not responsible for the Th1/Th2 switching effects observed in this experimental system. The influence of a polarized effector CD4 T cell, generated by activation of T cells in the periphery by crossreacting antigen, is largely ruled out by the finding that DO11.10 thymocytes, a vast majority of which are believed to be T cells not having experienced antigen in the periphery, are able to influence the XRBC-specific response in a similar manner as transgenic splenocytes, when adoptively transferred mice are challenged with XRBC-OVA.

That the effects could be attributed to a polarizing APC population, or to another population of polarizing non-T cells, is largely ruled out by the finding that highly purified transgenic CD4 T cells are able to influence the Th1/Th2 phenotype of the XRBC-OVA response, and with experiments showing that transgenic spleen cells depleted of CD4 T cells, and thus enriched for other putative pro-Th1 or pro-Th2 modulating cells, do not appreciatively influence the response phenotype.

That irradiated mice reconstituted with normal thymocytes, and challenged with XRBC-OVA, do not reliably generate a detectable immune response, in contrast to mice reconstituted with spleen cells, suggests an experimental approach to further define critical elements central to the Threshold Hypothesis. For example, the finding that reconstitution of irradiated mice with thymocytes and normal spleen cells depleted of T cells rescues antigen-specific responses, most likely indicates that an APC, which might be too limiting in the endogenous, irradiated splenocyte population of the irradiated host during the period of normal T cell activation, is critical in the activation of and Th1/Th2 commitment of naive CD4 T cells. A second possibility, suggested by the Two Step Two Signal Model's requirement for an antigen-specific effector T helper cell in the activation of naive T cells, is that lethally irradiated mice reconstituted with only thymocytes lack this subset. The finding that a population of CD44+ CD4 T cells (indicative of an activated phenotype) survives in the spleen of such mice at two days post-irradiation, combined with the verification, using different criteria, that T cell or CD4 T cell depleted spleen populations themselves do not generate XRBC-OVA-specific responses when used as reconstituting populations, supports the interpretation that a

lack of APC, and not a lack of effector T cells, is responsible for the ineffectiveness of thymocytes, by themselves, to generate detectable immune responses. In this respect it is perhaps worthwhile to briefly discuss the hypothesized role of the B cell in the Two Step Two Signal Model, upon which the Threshold Hypothesis is based.

6.2 The Role of the B cell in CD4 T cell activation/differentiation as envisioned by the Threshold Hypothesis

The Two Step Two Signal Model of CD4 T cell activation places critical importance on the interaction between an activated, antigen-specific B cell and the CD4 T cell to be activated (Bretscher, 1999). It is similarly thought that interaction between an antigen-specific B cell and the CD4 T cell to be activated is critical in determining the Th1/Th2 phenotype of the newly activated CD4 T cell. This model thus assumes that the same APC (type) is involved in both the activation of, and the initial Th1/Th2 polarization of naive CD4 T cells. However, it could be that antigen-specific B cells are required for the activation of, but not the Th1/Th2 polarization of CD4 T cells, or that B cells are only involved in the polarization of already activated CD4 T cells. In this case, a combination of different APC types would be required for the development of effector CD4 T cells, and the definition of exactly which APC populations persist in the lethally irradiated host is not addressed in this thesis. Indeed, the Two Step Two Signal Model proposes that DC/macrophages are generally involved in step one of CD4 T cell activation, and that B cells are involved in the second step, as discussed in 1.7.6. Importantly though, several different

studies support the proposal for a critical role for B cells in both the activation of, and Th1/Th2 commitment of CD4 T cells.

It is known that immunization with peptide antigen in CFA causes dendritic cells displaying the peptide to enter the draining lymph node and traffic to the T cell areas where naive T cells are restricted (Jenkins et al., 2001; Garside et al., 1998, Pape et al., 1998). Furthermore, it was found after immunization of non-irradiated mice into which were adoptively transferred a small number of OVA TCR transgenic CD4+ T cells, that these cells increased in number in the draining lymph node by 10-20 fold on day three after immunization compared to control, unimmunized mice. Following this initial burst of T cell activation, transgenic cells were found to move towards the B cell compartment; interestingly, the B cells themselves moved towards the T cells (Garside et al., 1998), suggesting that B cells are not simply passive antibody producing machines – for if the T cells move towards B cells to activate them, -although the production of antibody was not assessed in these experiments - then B cells might be moving towards T cells for a reason of their own. This highly repeatable pattern of lymph node trafficking is strikingly reminiscent of events predicted by the Two Step, Two Signal Model – the activation and initial increase in T cell numbers brought about by step one (mediated by an APC bearing constitutive costimulatory function, such as a DC) and the later interaction of these step one primed T cells and B cells, that, if they have been activated by the pre-existing eTh cells to express the vital signals needed, will allow the full activation of step one primed CD4 T cells to yield effector CD4 T cells.

Supporting evidence for this viewpoint has been obtained by the study of the chemokine receptor CXCR5, expressed by non-polarized T cells; CXCR5 is not expressed on resting CD4 T cells and expression is lost on fully differentiated CD4 T cells at sites of inflammation, although some exceptions to this strict CXCR5 expression pattern are seen in human T cells (Moser et al., 2002). Observations show that CXCR5 expression on CD4 T cells is upregulated following interaction with antigen on various DC subsets (Schaerli et al. 2001). It has been demonstrated that CXCR5 expression is responsible for the homing of a subset of $CXCR5^{+}$ CD4 T cells to B-cell areas of secondary lymphoid tissues in response to the chemokine BCA-1 (BLC in mice). Importantly, the expression of CXCR5 is " a common and early event in CD4 T cell activation ... that precedes T-cell proliferation and differentiation", thus serving as a marker for, "primed, but not yet polarized, Th cells with a limited proliferation history" (Moser et al., 2002). These observations are consistent with the Two Step Two Signal Model: following a 'step one' interaction with antigen presenting DCs in the T cell rich zones of secondary lymphoid organs, these step-one-primed CD4 T cells expressing CXCR5, enter the B cell rich follicles, where they potentially undergo 'step two' – assuming encounter with an antigen-specific B cell, presenting peptides for which the step one-primed CD4 T cell is specific. Although evidence supports a critical role for B cells in the full activation of naive T cells (Strutt, 2005) and potentially a role in Th1/Th2 regulation of activated CD4 T cells (Lund et al., 2005; Duddy et al., 2004; Harris et al., 2000), much work in this field remains to be done, especially as several experiments investigating the role of B cells in activating T cells *in vivo*, employing a variety of B

cell deficient mice, either by genetic engineering or by antibody depletion, are in some cases conflicting (for a thorough discussion of the complexities of these experimental systems, see Strutt, 2005). Beyond focusing only on B cell - T cell interactions or DC – T cell interactions in CD4 T cell priming, other recent studies have aimed at determining the relationships between DC's, B cells, and naive and effector T cells during T cell priming. For example, several pieces of evidence point towards models diametrically opposed to the linear "DC activate T cells which activate B cells" dogma, proposing instead that multiple "bilateral encounters" between these subsets, including direct DC – B cell interactions, are involved in important immunological activation events (reviewed in Ninio and Amigorena, 2004).

In opposition to this trend of investigating cooperation between cellular populations, some immunologists have proposed models explaining immunological coherence and independence in terms of properties of the DC. The *Eliminon* proposal, put forth by Langman and Cohn, suggests that the coherence and independence of immune responses, governed via "associative recognition", can be maintained by a DC, eliminating the necessity of antigen-specific B cells, as proposed by the Threshold Hypothesis (Langman and Cohn, 2000; Langman et al., 2003). In this model, the immune regulatory functions and self-non-self discrimination proposed in the Two Step Two Signal Model "can be maintained in situations where an APC, such as a DC, endocytoses two or more separate antigens, and presents peptides derived from the nominal antigens on its surface" by presenting peptides derived from separate nominal antigens each "in a confined

space or patch of the DC's surface" (Langman and Cohn, 2000). This mechanism could allow for antigen-specific T cell cooperation by the rearrangement of a specific antigen-presenting patch into a long, thin dendrite, physically removed from other antigen 'patches'. One recent report can be interpreted as showing that a DC loaded with both a strong Th1 and a separate, strong Th2 polarizing antigen can lead to the generation of separate, concurrent Th1 and Th2 antigen-specific responses (Cervi et al., 2004). Importantly though, if the activation requirements of naive CD4 T cells depend upon seeing both specific antigen and a costimulatory-dependent second signal, whose expression required the activation of the DC by an effector CD4 T helper cell, then a complete model based on the *Eliminon* proposal would seem to require an equally localized expression of this induced costimulatory molecule on the surface of the DC and/or localized activating/polarizing cytokines secretion, or would require direct signals transferred from an effector CD4 T cell to the naive CD4 T cell, brought in close (cell-surface) contact on a DC 'patch' displaying relevant antigen. It seems to me that evidence in support of these phenomena is lacking.

6.3 Future Directions

The experiments discussed in this thesis have investigated the impact of, and some details of the mechanism whereby CD4 T cell cooperation between antigen-specific precursors can influence the Th1/Th2 phenotype of the resulting CD4 effector T cells. The overriding hypothesis guiding this research, the Threshold Hypothesis, also envisions a critical importance for other cellular populations in generating and controlling responses - specifically, antigen-specific B cells, and pre-existing, antigen-specific effector CD4 T cells. Future directions of research, further investigating the Threshold Hypothesis, could logically focus on the role of these other cellular populations, and the molecular interactions between them, in determining Th1/Th2 phenotype. Finally, the reproduction of results presented in this thesis, employing a more defined adoptive transfer system, may alleviate concerns regarding possible unaccounted for interactions, which might impact the generation of immune responses. These points are elaborated on in the following subsections.

Although not discussed in detail, the adoptive transfer system described in this thesis could easily be employed to assess the impact of adding or removing any of a number of different cellular population (in addition to B cells) on the Th1/Th2 phenotype generated in response to a particular dose of antigen. For example, this approach could be used to investigate the involvement of antigen-specific CD8 T cells in the Th1/Th2 polarization of CD4 T cells (see 1.11.12). While not addressed in the experiments undertaken in this thesis, assessing the importance of CD8 T cells

and/or other cellular populations in this experimental system could offer a more complete description of cellular interactions critical in determining the Th1/Th2 phenotype of newly activated CD4 T cells.

6.3.1 Investigation on the role of the antigen-specific B cell in the Th1/Th2 decision criterion

Although a limited, preliminary interpretation of some results presented here suggests that B cells may be important in generating antigen-specific responses, and in maintaining coherence and independence between concurrent responses, this hypothesis requires more direct experimental evidence. In the design of future experiments, it should be kept in mind that the presence of B cells may be required for the proper reconstitution of the spleen and/or the maintenance of splenic architecture. For this reason, the preliminary results mentioned in this thesis, indicating differences in thymocyte responses between mice reconstituted with B cell (+) and B cell (-) populations, might be due to faulty/inefficient T cell reconstitution in mice receiving B cell (-) populations, and thus, may not be primarily due to inefficient T cell activation. Using B cell knockout mice as donors for reconstitution raises the same concerns, and also, certain knockout strains appear to allow for 'leaky' B cell expression, making interpretations employing these mice complicated (see Strutt, 2005 for a thorough discussion).

A more elegant approach to investigate the role that *antigen-specific B cells* might serve in Th1/Th2 polarization of naive CD4 T cells, would be the use of a B

cell donor mouse transgenic for Ig specific for an irrelevant antigen, for example HEL-specific B cells (Goodnow, 1988) to reconstitute lethally irradiated mice together with a population of normal, syngeneic thymocytes, and challenge with XRBC-OVA. This system would ideally be suited to investigate whether *antigen-specific* B cells are required to generate either Th1, Th2, or both responses. If this population of transgenic B cells gives rise to significant differences than do normal B cells, then normal B cells could potentially be titrated into the transgenic reconstituting population in an effort to restore the control response. As mentioned, by adjusting other variables, such as CD4 T cell number in the reconstituting population, or antigen dose, it could be investigated whether antigen-specific B cells are critically involved in both Th1 and Th2 responses, or whether this population is more critically involved in either a Th1 or Th2 response. Similarly, it is perhaps possible to discriminate, in this adoptive transfer system, between models of coherence/independence based on antigen-specific B cells and DC-based models, such as the Eliminon Hypothesis discussed earlier.

6.3.2 Investigation on the role of pre-existing antigen-specific effector CD4 T cells in Th1/Th2 differentiation

Radio-resistant effector CD4 T cells (Kettman and Dutton, 1971; Hamaoka et al., 1972) persist in the spleens of lethally irradiated mice during the initiation phases of responses in this adoptive transfer system (and possibly survive longer, data not shown). To evaluate the importance of this population in either or both the processes

of CD4 T cell activation and Th1/Th2 polarization, a similar adoptive transfer to that used in the current experiments, can be employed.

It is expected that this population will be eliminated in lethally irradiated, thymectomized, and bone marrow-reconstituted mice, and resting these mice for two months or so. The role of pre-existing effector CD4 T cells can be assayed *in vivo* by comparing the responses generated in adoptive transfer hosts, treated as described above, compared to sham-thymectomized, irradiated and bone marrow-reconstituted hosts, challenged with the same antigen. By immunizing and reconstituting these hosts in either a defined pro-Th1 or pro-Th1/Th2 manner, it could be investigated whether effector CD4 T cells are involved in the generation of either Th1 or Th2, or both response phenotypes.

6.3.3 Costimulatory molecules/cytokines involved in CD4 T cell cooperative events influencing the Th1/Th2 phenotype of a primary immune response

The steps and signals, and the nature of the cooperative events described in the Two Step Two Signal Model, and in the Threshold Hypothesis are, to a large extent, undefined molecularly. In fact, each step, each signal, each cooperative event proposed might be made up of several individual sub-steps – either sequential or additive in nature. If the cellular populations involved in determining the Th1/Th2 phenotype of newly activated CD4 T cells, and if their interactions, can be determined, then the stage would be set for incisive investigation of pertinent

intercellular signaling molecules involved in the various steps and cellular interactions determining Th1/Th2 phenotype.

To investigate the critical molecular signals involved in the Th1/Th2 decision criterion, one could employ as reconstituting populations in the adoptive transfer system, cells from donor mice engineered to lack expression of defined cell-surface costimulatory molecules, receptors, or cytokines. This approach could conceivably allow for the control of signals derived from separate reconstituting populations concurrently, by employing, for example, different specific knockout sources for reconstituting APC, thymocytes, effector T cells, and perhaps even knockout hosts. This approach could lead to a significantly more detailed understanding of the inducible and constitutive costimulatory molecules, and perhaps, cytokines, involved in the Th1/Th2 differentiation of naive CD4 T cells.

An alternative approach to dissecting signals critical in either or both Th1/Th2 maturation is the development of an *in vitro* system, in which the same variables of dose and CD4 T cell number are found to influence Th1/Th2 phenotype as described in this thesis *in vivo*. Preliminary work has been done in the development of such as system (data not shown). If DO11.10 CD4 T cells can be employed to alter the XRBC-specific response when added to wells containing normal spleen cells and the antigen XRBC-OVA, then it is possible that blocking antibodies directed against specific cell-surface molecules/cytokines could be used to assess the importance of specific signals in altering the response phenotypes observed.

6.3.4 Other antigens and sources of antigen-specific CD4 T cells

It could be advantageous to repeat the central experiments described in this thesis using an adoptive transfer system, with a source of transgenic CD4 T cells on a RAG $^{-/-}$ background. Such a source would minimize many unaccounted for influences - which might affect the outcomes of experiments employing mice not on a RAG $^{-/-}$ background - of transgenic T cells specific for a different antigen than the transgenic specificity, and perhaps in various other (not naive) activation states, because of previous encounter with such antigens, including intestinal flora (Smiley and Grusby, 1997; Saparov et al., 1999; Zhou et al., 2004). In DO11.10 mice on a RAG $^{+/+}$ background, up to 10% of clonotypic TCR-positive CD4 T cells isolated from the spleens of naïve animals express an activated phenotype, and a small but detectable population of CD8 T cells can be found to express the transgenic TCR (Pape et al., 1997). By using a RAG $^{-/-}$ DO11.10 strain, some of these concerns can be addressed.

To avoid the concern raised by Matzinger (Anderson and Matzinger, 2000), that XRBC might not represent an antigen against which a pristine, primary immune response can be generated (see next section), it might be possible to employ as antigen a combination of different purified simple proteins, made immunogenic without adjuvant. One possibility is to aggregate preparations of HEL and OVA proteins together, creating an immunogenic HEL-OVA conjugate. If conditions in the adoptive transfer system can be defined in which Th1 responses can be generated against HEL-OVA, then it could be tested whether the addition of DO11.10 CD4 T

cells could influence the response phenotype of the HEL-specific response in a similar manner as has been presented in this thesis. An additional level of investigation in this system could be developed by the use of defined HEL peptides involved in the BALB/c HEL-specific response (see Peters, 2003) in the ELISPOT assay – enabling a finer investigation of discrete responding CD4 T cell populations.

6.4 Concluding Remarks

The experiments discussed in this thesis support a model in which the Th1/Th2 phenotype of a newly activated CD4 T cell is determined by a cooperative mechanism, influenced by quantitative variables. Specifically, cellular interactions between CD4 T cells, mediated by the recognition of specific antigen, can determine the immune response phenotype: optimal cooperation, in situations where neither antigen nor specific CD4 T cells are limiting, resulting in predominant Th2 responses, and Th1-like responses generated in situations of sub-optimal cooperative events because of either limiting antigen, or limiting numbers of specific CD4 T cells, or both. This Th1/Th2 phenotype decision criterion can account for several variables of immunization long known to impact the cellular/humoral character of the immune response (see 1.10). The Threshold Hypothesis, which these observations support, is also formulated on teleological grounds, relating the degree of foreignness of an antigen and the effector mechanism employed by the immune system for effective clearance (see 2.0).

Competing models describing the Th1/Th2 decision criterion are largely based on professional APC populations, most notably DC's, and their recognition of foreign antigen by virtue of evolutionarily conserved, germline encoded pattern recognition receptors that, upon recognizing their specific pattern(s), ultimately control the Th1/Th2 polarizing potential of an APC presenting peptides derived from the recognized pattern-containing antigen. Such models, which ascribe minimal importance to cells of the adaptive immune system in influencing Th1/Th2

commitment of naive helper cells, forcefully *date* Nossal's (then) well-accepted generalization: "lets put it this way: lymphocytes are clever, macrophages [APC in general] are stupid" (Sterzl and Riha, 1969).

It is true, that great advances have been made in the past decade or so, which have dispelled the long-standing belief that the cells of the innate defense system, particularly DCs, are 'stupid' – without much influence over the brain-trust of the adaptive immune system. But more recently, the pendulum of immunological dogma has swung resoundingly against Nossal's point of view to, in the case of the Th1/Th2 decision criterion, almost labeling adaptive lymphocytes as 'stupid', - or in other (softer) words, not involved in the decision of effector phenotype. This shift is at least in part due to the emergence of Matzinger's Danger Model, and, more importantly, Janeway's '*Stranger*' Model of T cell activation (see 1.7.1 and 1.7.3), which both place extreme emphasis on the recognition of antigen (or adjuvant) by elements of the body's innate defense system.

There are, however, several examples of primary immune responses generated in the absence of overt adjuvants, with antigens unlikely to otherwise trigger conserved pattern recognition receptors, including XRBC-specific responses, responses generated against alloantigens, and responses generated against aggregated proteins, such as HEL (Peters, 2003). Such observations lead one to call into question the validity of models of T cell activation *requiring* a 'dirty little secret' – Silverstein and Rose, citing observations of very good T cell-dependent responses made in the 1950's and 1960's generated against non-microbial antigens, state that "too much is made these days of the critical importance of adjuvants" (Silverstein

and Rose, 2000). This in turn suggests that elements of the innate defense system are not obligatory in the Th1/Th2 decision either, and further suggests the potential that, even in the presence of microbial products or danger signals, variables proposed by the Threshold Hypothesis can impact the phenotype of specific responses. As discussed earlier, this view is supported by observations made with several pathogenic and other microbial organisms, in that the challenge dose can still significantly affect Th1/Th2 phenotype.

This is not to say that adjuvants or microbial/danger signals cannot impact upon the Th1/Th2 decision criterion – a wealth of clinical and experimental data attests to this. Furthermore, it is to be expected on evolutionary grounds that protective elements of earlier defense mechanisms, conserved in evolutionarily higher animals with adaptive immune systems, would contribute in a meaningful way, and not simply be carried as baggage or as emergency back-ups. Apart from their role in the initiation events of an immune response, as proposed in the *danger/stranger* model, it has been proposed that pattern recognition receptors might instead be exclusively involved in feedback regulation of ongoing immune responses, releasing pro-Th1/Th2 cytokines and expressing costimulatory molecules *after* detecting "scalps" – components of killed invaders "that signal its destruction", such as LPS or CpG DNA, "up-regulate the differentiation of the appropriate T helper subset that is responsible for the destruction" (Bergmann et al., 2002). Because the cells and receptors of the innate defense system have, to a great extent, not been directly addressed in the experiments described here, it should not be concluded that their presence and impact is entirely dismissed. To return once more

to Hesse, I think that the core of the Threshold Hypothesis, and variables found to influence the Th1/Th2 polarizing capabilities of innate immune cells, can in many situations be integrated, for example, in vaccine formulations and in cancer treatment.

As the study of the history of the science of immunology reveals, and, as the vertebrate immune system itself has no doubt evolved, the integration of individual elements or approaches often leads to entirely new solutions. The Th1/Th2 decision criterion remains an *Indian Elephant*, if you will, but surrounded by considerably more than just six individuals. In a way, the activity of science tends to blind one – so much effort is spent on one's own research that it is often difficult, especially in today's environment of almost endless journal titles, to properly 'see' competing points of view. Perhaps then, we must content ourselves with always to be only "partly in the right". This realization is a prerequisite for healthy discussion and collaboration whereby, together, even blind men can reach a powerful consensus.

6.5 Interpretations: touching the elephant

6.5.1 The effect of cell density on the Th1/Th2 phenotype

As introduced by the parable discussed at the beginning of this thesis, individual observations, when judged in isolation, can lead to very different interpretations. As mentioned earlier (1.10.7), studies done in the 1980's using *in vitro* systems supported the conclusion that the density of CD4 T cells in culture could significantly influence the Th1/Th2 phenotype of effector cells generated in these cultures (Bretscher, 1983; Bretscher, 1986). Similar observations have recently been made by a different laboratory, though not published in detail:

> ... we observed the quite artificial influence in vitro of the density of T cells on the polarization of naive CD4 T cells ... We observed that, while keeping the number of APC and OVA peptide constant, lower densities of $CD4^+$ DO11.10 Tg T cells generally led to more of a Th1-type response, whereas increased densities of DO11.10 Tg T cells led to more of a Th2-type phenotype. While initially perplexing, this effect is now understood as likely resulting from the changing concentrations of initial IL-4 produced by activated T cells early in the culture, with higher numbers of T cells leading to higher levels of IL-4 capable of recruiting T cells towards the Th2 phenotype ... At low numbers of T cells, IL-12 and IL-18 produced by APC were likely in relative abundance over IL-4 produced by T cells, making a more Th1-like response ... Thus, while it is true that T cell density influences Th1/Th2 polarization in vitro, we hesitated to conclude that precursor frequency controls Th1/Th2 development (Berenson et al., 2004)

I was excited to read this passage because, not only is it supportive of the Threshold Hypothesis, but it also offers an opportunity to reintroduce the moral of Saxe's poem. These observations were most probably considered "perplexing" because they did not fit into the laboratory's governing hypothetical research framework. When viewed in isolation, the explanation put forth by the authors from Murphy's laboratory offers an interesting and equally sound explanation for the effects of precursor CD4 T cell frequency and the response phenotype generated, as does the Threshold Hypothesis. *In vitro*, cellular populations, and their secreted products, are bounded in a restricted space, potentially giving rise to unphysiological concentrations of specific cytokines, which might affect Th1/Th2 polarization in a profound manner. Indeed, many subtle and not so subtle differences exist regarding the activation of T cells *in vitro* and *in vivo* (Fazekas de St. Groth et al., 2004). However, when viewed in combination with a body of *in vivo* experiments – specifically adoptive transfer systems, which have revealed the same relationship between the number of responding T cells and immune response phenotype (as presented in this thesis, as well as Bretscher, 1983; Ismail and Bretscher, 2001) then a *purely* cytokine driven model of Th1/Th2 differentiation, as proposed by Murphy and colleagues, seems more unlikely; *in vivo*, the complex trafficking of different cellular populations, and opportunity for soluble factors to 'move' - unbounded by plastic – makes this type of model, dependent strictly on a fine balance between opposing cytokines, unappealing, especially when imagining situations of two or more concurrent antigen challenges, requiring separate Th1/Th2 responses for effective clearance.

6.5.2 A primary immune response?

Critical in the construction of a valid experimental system for investigating the activation of and differentiation of naive T cells – events associated with a primary immune response - is the use of an antigen(s) which naive mice have not previously been exposed to. Polly Matzinger has suggested that the SRBC-specific immune response in mice might not represent a true 'primary' immune response (Anderson and Matzinger, 2000). She bases this statement on observations that, in the spleen of unimmunized mice, several 'background' plaques are observed when SRBC is used as antigen in a plaque-forming assay; presumably this reflects "ongoing antigen-dependent stimulation in normal mice" (Bretscher, 1978) – most likely due, in Matzinger's view, to a crossreacting environmental antigen. The presence of antibody directed against SRBC in a naive mouse allows for the interpretation that antigen-antibody complexes, recognized by and ingested via DC Fc receptors, replace the 'danger signal', which was originally critical in initiating the SRBC-specific response, as "once the immune system has encountered a dangerous pathogen, it does not need to wait for damage the second time" (Anderson and Matzinger, 2000). While this might be a sound criticism of employing SRBC (or CRBC for that matter – though Matzinger's statement introduces profound problems for her model) when wanting to analyzing a primary response, in Bretscher's 1978 study, robust primary responses were generated in mice against rat RBC and pig RBC, for which very few (pig RBC) or virtually no (rat RBC) specific

background plaques were observed. Thus, while the murine anti-SRBC immune response is in a certain manner unique, considering the relatively high number of background antibody-producing cells observed, this unique characteristic of the response does not seem to be a requirement for the generation of XRBC-specific immune responses in general. On the other hand, part of the *human condition* itself is that we are all and always exposed to a myriad of antigens, and it is therefore likely that some degree of cross-reactivity exists between theoretically 'non-immune' sera and virtually every antigen that impinges on such a 'dirty' immune system. As stated earlier, the advantages of using XRBC as antigen in this system are that it represents a relatively complex, non-replicating antigen, immunogenic when given without adjuvants, which does not display overt immunomodulatory activity, making it well suited to address the research goals of this thesis project.

6.3.3 Variables affecting Th1/Th2 phenotype: comparison or integration

In a biological process as complex and regulated as that involed in CD4 T cell activation and Th1/Th2 phenotype polarization, it would not be suprising to find that several important variables can influence decisions at several different time points. The literature, as concerns Th1/Th2 differentiation of CD4 T cells, certainly validates this point of view (see Introduction 1.11). Which variables are the most important in influencing this decision?

One approach to ordering the many variables and factors found to impact Th1/Th2 phenotype in a list of importance could be an attempt to place them in a chain of causality. For example, is it less likely that the production of a pro-Th1 or pro-Th2 cytokine could influence the dose of antigen, as seen by an antigen-specific CD4 T cell, than it is that the dose of antigen, seen by a CD4 T cell, could influence the production of pro-Th1 or pro-Th2 cytokines? Another approach could be to determine which variables have the most profound effect on Th1/Th2 phenotype. For example, in most cases, the effects of a polarizing adjuvant probably exerts a more sweeping effect on the immune response than the depletion of a certain APC subtype or the deletion of a particular costimulatory molecule. Yet another approach could be to try and determine which variables can be omitted from the Th1/Th2 equation while still describing the phenotype of the response generated – assigning more importance to certain variables (genetic background), less to others (the individual's amount of sleep or nutritional status).

This kind of mental play, using the data summarized in 1.11, is reminicent of the blind men in Saxe's poem, arguing over what they 'see', - each observer feeling theirs alone is *the* critical observation. But what is more an elephant? The trunk or an ear, the tail or a leg? What is more important in swaying Th1/Th2 phenotype - the antigen dose, recognition of danger/stranger signals, or the general cytokine milieu? I believe that the work presented in this thesis offers a framework for understanding many aspects of Th1/Th2 regulation, but not all in every situation (take, for example, the wealth of data concerning peptide dose and its effect on Th1/Th2 phenotype).

To be sure, this thesis has concentrated on, and placed primary importance with certain variables. But, I do believe that finding meaningful connections between variables and observations, and not assigning rank or importance to them, is the surest way to increase understanding – be it understanding of the Th1/Th2 decision criterion, or of an elephant.

7.0 References

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